

File Number : 00-81
Filing Date: August 29, 2001
Express Mail Label No. EL547696636US

TO DO NOT FILE THIS DOCUMENT

UNITED STATES PATENT APPLICATION

OF

James L. Holloway, Zeren Gao, Paul D. Bishop

FOR

MAMMALIAN CYSTATIN-8 AND ITS USE TO INHIBIT CANCER PROCOAGULANT

PROTEIN

MAMMALIAN CYSTATIN-8 AND ITS USE TO INHIBIT
CANCER PROCOAGULANT PROTEIN

5

This claims the benefit of United States Provisional Application No.
60/230,230 filed September 1, 2001.

BACKGROUND OF THE INVENTION

10

The cystatin superfamily is an evolutionarily related group of proteins consisting of at least three families, i.e., stefins (type 1), cystatins (type 2), and kininogens (type 3). See, for example, Barrett, *TIBS* 12: 193-196 (1987). Generally, stefin family members are unglycosylated proteins consisting of about 100 amino acids that are devoid of disulfide bonds. In contrast, cystatin family members are proteins consisting of about 115 amino acids and characterized by two disulfide bonds in the carboxy-terminal region of the protein. Finally, kininogens contain three regions containing two disulfide loops, similar to the carboxy terminal domain found in members of the cystatin family. The cystatin superfamily are inhibitors of cysteine proteinases (also referred to as cysteine proteases) and are believed to function in a protective role with regard to pathological action of endogenous or exogenous cysteine proteinases. It is believed that cystatins form equimolar reversible complexes with cysteine proteinases.

Cystatin-like proteins have also been identified. One such protein, cystatin-related epididymal specific gene (CRES) does not contain the conserved sequence motifs necessary for cysteine proteinase inhibitory activity [Cornwell et al., *Mol. Endocrinol.* 6:1653-64 (1992) and Cornwell and Hann, *Mol. Reprod. Dev.* 41:37-46, (1995)]. Also, unlike the ubiquitous expression of many of the cystatins, CRES is restricted to the proximal caput epididymal epithelium and testis. CRES expression is stage specific during spermatogenesis and CRES is found in both round and elongating spermatids suggesting a specialized role during spermatogenesis. Cystatins are also

TOP SECRET - 100

found with male reproductive tissues and secretions. Cystatin C for example is found in highest abundance in human semen and participates in spermatogenesis and spermogenesis and is associated with the sperm throughout its time in the male genital tract [Esnard *et al.*, *FEBS Lett.* 300:131-5, (1992)]. Testatin is believed to be involved
5 in early testis development. Expression is restricted to fetal gonads and adult testis and it is expressed during testis cord formation in pre-Sertoli cells [Töhönen *et al.*, *Proc. Natl. Acad. Sci. USA* 95:14208-13 (1998)].

Proteins capable of modulating spermatogenesis are sought for the study, diagnosis and treatment of conditions associated with reproductive disorders. There is
10 also a need to discover proteins that can inhibit cancer procoagulant protein. Cancer procoagulant protein is a cysteine protease that activates factor X, and thereby causing a thrombotic condition in a number of cancers including acute promyelocytic leukemia. The present invention provides such polypeptides for these and other uses that should be apparent to those skilled in the art from the teachings herein.
15

DESCRIPTION OF THE INVENTION

The present invention fills this need by providing for a novel cystatin, designated "mammalian cystatin-8", hereinafter referred to as "Zcys8". The present
20 invention also provides Zcys8 polypeptides and Zcys8 fusion proteins, as well as nucleic acid molecules encoding such polypeptides and proteins.

In particular, the present invention provides isolated polypeptides having an amino acid sequence that is at least 90% identical to an amino acid sequence selected from the group consisting of (a) SEQ ID NO: 2, wherein the isolated
25 polypeptide either specifically binds with an antibody that specifically binds with a polypeptide having the amino acid sequence of either SEQ ID NO:2.

The present invention further provides pharmaceutical compositions that comprise such polypeptides, and a pharmaceutically acceptable carrier.

The present invention further provides isolated nucleic acid molecules
30 that encode the above-described polypeptides. In particular the present invention

T062800-11111111

provides for nucleic acid molecules comprising the nucleotide sequence of nucleotides of SEQ ID NO: 1.

The present invention also provides vectors and expression vectors comprising such nucleic acid molecules, recombinant host cells comprising such 5 vectors and expression vectors, and recombinant viruses comprising such expression vectors. These expression vectors and recombinant host cells can be used to prepare Zcys8 polypeptides. In addition, the present invention provides pharmaceutical compositions, comprising a pharmaceutically acceptable carrier and at least one of such an expression vector or recombinant virus. Preferably, such pharmaceutical 10 compositions comprise a human Zcys8 gene, or a variant thereof.

The present invention further contemplates antibodies and antibody fragments that specifically bind with Zcys8 polypeptides. Such antibodies include polyclonal antibodies, murine monoclonal antibodies, humanized antibodies derived from murine monoclonal antibodies, and human monoclonal antibodies. Examples of 15 antibody fragments include F(ab')₂, F(ab)₂, Fab', Fab, Fv, scFv, and minimal recognition units.

The present invention also provides methods for detecting the presence of Zcys8 RNA in a biological sample, comprising the steps of :

- (a) contacting an Zcys8 nucleic acid probe under hybridizing conditions 20 with either (i) test RNA molecules isolated from the biological sample, or (ii) nucleic acid molecules synthesized from the isolated RNA molecules, wherein the probe has a nucleotide sequence comprising a portion of the nucleotide sequence selected from the group consisting of SEQ ID NO:1, or the complement of SEQ ID NO:1, and
- (b) detecting the formation of hybrids of the nucleic acid probe and either 25 the test RNA molecules or the synthesized nucleic acid molecules,

wherein the presence of the hybrids indicates the presence of Zcys8 RNA in the biological sample.

In addition, the presence of Zcys8 polypeptide in a biological sample can be detected by methods that comprise the steps of (a) contacting the biological sample 30 with an antibody, or an antibody fragment, that specifically binds with a polypeptide having the amino acid sequence of either SEQ ID NOs: 2 - 17 wherein the contacting is

performed under conditions that allow the binding of the antibody or antibody fragment to the biological sample, and (b) detecting any of the bound antibody or bound antibody fragment.

The present invention also provides kits for detecting Zcys8 nucleic acid molecules or Zcys8 polypeptides. For example, a kit for detection of Zcys8 nucleic acid molecules may comprise a container that comprises a nucleic acid molecule, wherein the nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1, (b) a nucleic acid molecule comprising the complement of the nucleotide sequence of SEQ ID NO: 1, (c) 10 a nucleic acid molecule that is a fragment of (a) consisting of at least eight nucleotides, (d) a nucleic acid molecule that is a fragment of (b) consisting of at least eight nucleotides, (e) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1. Such kits may further comprise a second container that comprises one or more reagents capable of indicating the presence of the nucleic acid molecule. A kit for 15 detection of Zcys8 polypeptide may comprise a container that comprises an antibody, or an antibody fragment, that specifically binds with a polypeptide having the amino acid sequence of either SEQ ID NOs: 2 – 17.

An additional embodiment of the present invention relates to a peptide or polypeptide that has the amino acid sequence of an epitope-bearing portion of a 20 Zcys8 polypeptide having an amino acid sequence described above. Peptides or polypeptides having the amino acid sequence of an epitope-bearing portion of a Zcys8 polypeptide of the present invention include portions of such polypeptides with at least nine, preferably at least 15 and more preferably at least 30, 40 to 50 amino acids, although epitope-bearing polypeptides of any length up to and including the entire 25 amino acid sequence of a polypeptide of the present invention described above are also included in the present invention. Examples of said polypeptides are defined by the amino acid sequences of SEQ ID NOs: 2-17. Also claimed are any of these polypeptides that are fused to another polypeptide or carrier molecule.

The invention provides an isolated polynucleotide molecule that encodes 30 a polypeptide, wherein said polypeptide comprises a sequence of amino acid residues that is selected from the group consisting of: a) a sequence of amino acid residues that

TROG2000-17660

is 80% identical to the amino acid sequence of SEQ ID NO:2, and wherein said polypeptide comprising said amino acid sequence specifically binds with an antibody that specifically binds with a polypeptide having the amino acid sequence of SEQ ID NO:2; b) degenerate nucleotide sequence of a); c) nucleotide sequences complementary 5 to a) or b); and d) a sequence of amino acid residues encoded by a polynucleotide sequence which hybridizes under stringent conditions to a similarly sized polynucleotide sequence of SEQ ID NO:1. Within one embodiment any difference between said amino acid sequence encoded by said polynucleotide molecule and said corresponding amino acid sequence of SEQ ID NO:2 is due to one or more 10 conservative amino acid substitutions. Within another embodiment the amino acid percent identity is determined using a FASTA program with ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=blosum62, with other parameters set as default. Within yet another embodiment the polypeptide further comprises an affinity tag or binding domain.

15 The present invention also contemplates anti-idiotype antibodies, or anti-idiotype antibody fragments, that specifically bind with an anti-Zcys8 antibody or antibody fragment.

The teachings of all of the references cited herein are incorporated in their entirety herein by reference.

20

1. *Introduction*

The cystatin superfamily is subdivided into stefins, cystatins and kininogens. Stefins are single chain proteins of M_r of about 11,000 that lack disulfide bonds and carbohydrates. Cystatins are single chain proteins characterized by the 25 presence of two disulfide bonds in the carboxy terminal region thereof. Moreover, cystatins are composed of about 115 amino acids with M_r of about 13,000. Some cystatins exhibit phosphorylated and dephosphorylated forms. Kininogens are higher molecular weight moieties that contain three regions of homology to cystatins (*i.e.*, three sets of two disulfide loops); however, only two of such regions are believed to be 30 active. See, for example, Turk and Bode, *FEBS Lett.* 285(2):213-9 (1991); Barrett,

DRAFT - 08/20/01

TIBS 12: 193-6 (1987); and Bobek and Levine, *Crit. Rev. Oral Biol. Med.* 3: 307-32 (1992).

2. Definitions

In the description that follows, a number of terms are used extensively.

- 5 The following definitions are provided to facilitate understanding of the invention.

As used herein, "nucleic acid" or "nucleic acid molecule" refers to polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease 10 action. Nucleic acid molecules can be composed of monomers that are naturally- occurring nucleotides (such as DNA and RNA), or analogs of naturally occurring nucleotides (*e.g.*, α -enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for 15 example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated 20 purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. The term "nucleic acid molecule" also includes so- 25 called "peptide nucleic acids," which comprise naturally occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded.

The term "complement of a nucleic acid molecule" refers to a nucleic acid molecule having a complementary nucleotide sequence and reverse orientation as 30 compared to a reference nucleotide sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term “contig” denotes a nucleic acid molecule that has a contiguous stretch of identical or complementary sequence to another nucleic acid molecule. Contiguous sequences are said to “overlap” a given stretch of a nucleic acid molecule either in their entirety or along a partial stretch of the nucleic acid molecule.

5 The term “degenerate nucleotide sequence” denotes a sequence of nucleotides that includes one or more degenerate codons as compared to a reference nucleic acid molecule that encodes a polypeptide. Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (*i.e.*, GAU and GAC triplets each encode Asp).

10 The term “structural gene” refers to a nucleic acid molecule that is transcribed into messenger RNA (mRNA), which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

15 An “isolated nucleic acid molecule” is a nucleic acid molecule that is not integrated in the genomic DNA of an organism. For example, a DNA molecule that encodes a growth factor that has been separated from the genomic DNA of a cell is an isolated DNA molecule. Another example of an isolated nucleic acid molecule is a chemically synthesized nucleic acid molecule that is not integrated in the genome of an organism. A nucleic acid molecule that has been isolated from a particular species is smaller than the complete DNA molecule of a chromosome from that species.

20 A “nucleic acid molecule construct” is a nucleic acid molecule, either single- or double-stranded, that has been modified through human intervention to contain segments of nucleic acid combined and juxtaposed in an arrangement not existing in nature.

25 “Linear DNA” denotes non-circular DNA molecules having free 5' and 3' ends. Linear DNA can be prepared from closed circular DNA molecules, such as plasmids, by enzymatic digestion or physical disruption.

30 “Complementary DNA (cDNA)” is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term “cDNA” to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its

TOP SECRET - DEFENSE

complementary DNA strand. The term "cDNA" also refers to a clone of a cDNA molecule synthesized from an RNA template.

A "promoter" is a nucleotide sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5' non-coding region of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These promoter elements include RNA polymerase binding sites, TATA sequences, CAAT sequences, differentiation-specific elements [DSEs; McGehee *et al.*, *Mol. Endocrinol.* 7:551 (1993)], cyclic AMP response elements (CREs), serum response elements [SREs; Treisman, *Seminars in Cancer Biol.* 1:47 (1990)], glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF [O'Reilly *et al.*, *J. Biol. Chem.* 267:19938 (1992)], AP2 [Ye *et al.*, *J. Biol. Chem.* 269:25728 (1994)], SP1, cAMP response element binding protein (CREB; Loeken, *Gene Expr.* 3:253 (1993)) and octamer factors [see, in general, Watson *et al.*, eds., *Molecular Biology of the Gene*, 4th ed. (The Benjamin/Cummings Publishing Company, Inc. 1987), and Lemaigre and Rousseau, *Biochem. J.* 303:1 (1994)]. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Repressible promoters are also known.

A "core promoter" contains essential nucleotide sequences for promoter function, including the TATA box and start of transcription. By this definition, a core promoter may or may not have detectable activity in the absence of specific sequences that may enhance the activity or confer tissue specific activity.

A "regulatory element" is a nucleotide sequence that modulates the activity of a core promoter. For example, a regulatory element may contain a nucleotide sequence that binds with cellular factors enabling transcription exclusively or preferentially in particular cells, tissues, or organelles. These types of regulatory elements are normally associated with genes that are expressed in a "cell-specific," "tissue-specific," or "organelle-specific" manner. For example, the Zcys8 ϵ regulatory element preferentially induces gene expression in placental, tracheal, and uterine

tissues, as opposed to lung, brain, liver, kidney, spleen, thymus, prostate, testis, ovary, small intestine, and pancreas tissues.

An “enhancer” is a type of regulatory element that can increase the efficiency of transcription, regardless of the distance or orientation of the enhancer relative to the start site of transcription.

“Heterologous DNA” refers to a DNA molecule, or a population of DNA molecules, that does not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species (*i.e.*, endogenous DNA) so long as that host DNA is combined with non-host

DNA (*i.e.*, exogenous DNA). For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a transcription promoter is considered to be a heterologous DNA molecule. Conversely, a heterologous DNA molecule can comprise an endogenous gene operably linked with an exogenous promoter. As another illustration, a DNA molecule comprising a gene derived from a wild-type cell is considered to be heterologous DNA if that DNA molecule is introduced into a mutant cell that lacks the wild-type gene.

A “polypeptide” is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as “peptides.”

A “protein” is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

A peptide or polypeptide encoded by a non-host DNA molecule is a “heterologous” peptide or polypeptide.

An “integrated genetic element” is a segment of DNA that has been incorporated into a chromosome of a host cell after that element is introduced into the cell through human manipulation. Within the present invention, integrated genetic

elements are most commonly derived from linearized plasmids that are introduced into the cells by electroporation or other techniques. Integrated genetic elements are passed from the original host cell to its progeny.

A “cloning vector” is a nucleic acid molecule, such as a plasmid, cosmid, or bacteriophage that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites that allow insertion of a nucleic acid molecule in a determinable fashion without loss of an essential biological function of the vector, as well as nucleotide sequences encoding a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

An “expression vector” is a nucleic acid molecule encoding a gene that is expressed in a host cell. Typically, an expression vector comprises a transcription promoter, a gene, and a transcription terminator. Gene expression is usually placed under the control of a promoter, and such a gene is said to be “operably linked to” the promoter. Similarly, a regulatory element and a core promoter are operably linked if the regulatory element modulates the activity of the core promoter.

A “recombinant host” is a cell that contains a heterologous nucleic acid molecule, such as a cloning vector or expression vector. In the present context, an example of a recombinant host is a cell that produces Zcys8 from an expression vector. In contrast, Zcys8 can be produced by a cell that is a “natural source” of Zcys8, and that lacks an expression vector.

“Integrative transformants” are recombinant host cells, in which heterologous DNA has become integrated into the genomic DNA of the cells.

A “fusion protein” is a hybrid protein expressed by a nucleic acid molecule comprising nucleotide sequences of at least two genes. For example, a fusion protein can comprise at least part of a Zcys8 polypeptide fused with a polypeptide that binds an affinity matrix. Such a fusion protein provides a means to isolate large quantities of Zcys8 using affinity chromatography.

The term “receptor” denotes a cell-associated protein that binds to a bioactive molecule termed a “ligand.” This interaction mediates the effect of the ligand

on the cell. Receptors can be membrane bound, cytosolic or nuclear; monomeric (*e.g.*, thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (*e.g.*, PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor). Membrane-bound receptors are
5 characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. In certain membrane-bound receptors, the extracellular ligand-binding domain and the intracellular effector domain are located in separate polypeptides that comprise the complete functional receptor.

10 In general, the binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell, which in turn leads to an alteration in the metabolism of the cell. Metabolic events that are often linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production,
15 mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids.

The term “secretory signal sequence” denotes a DNA sequence that encodes a peptide (a “secretory peptide”) that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is
20 synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

An “isolated polypeptide” is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Typically, a preparation of isolated
25 polypeptide contains the polypeptide in a highly purified form, *i.e.*, at least about 80% pure, at least about 90% pure, at least about 95% pure, greater than 95% pure, or greater than 99% pure. One way to show that a particular protein preparation contains an isolated polypeptide is by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and
30 Coomassie Brilliant Blue staining of the gel. However, the term “isolated” does not

SEARCHED
INDEXED
SERIALIZED
FILED

exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The terms "amino-terminal or N-terminal" and "carboxyl-terminal or C-terminal" are used herein to denote positions within polypeptides. Where the context

5 allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term “expression” refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a polypeptide encoded by a splice variant of an mRNA transcribed from a gene.

As used herein, the term "immunomodulator" includes cytokines, stem cell growth factors, lymphotoxins, co-stimulatory molecules, hematopoietic factors, and synthetic analogs of these molecules.

The term “complement/anti-complement pair” denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions.

25 For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement

30 pair preferably has a binding affinity of less than 10^9 M^{-1} .

An “anti-idiotype antibody” is an antibody that binds with the variable region domain of an immunoglobulin. In the present context, an anti-idiotype antibody binds with the variable region of an anti-Zcys8 antibody, and thus, an anti-idiotype antibody mimics an epitope of Zcys8.

5 An “antibody fragment” is a portion of an antibody such as F(ab')₂, F(ab)₂, Fab', Fab, and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. For example, an anti-Zcys8 monoclonal antibody fragment binds with an epitope of Zcys8.

The term “antibody fragment” also includes a synthetic or a genetically
10 engineered polypeptide that binds to a specific antigen, such as polypeptides consisting of the light chain variable region, “Fv” fragments consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker (“scFv proteins”), and minimal recognition units consisting of the amino acid residues that mimic the
15 hypervariable region.

A “chimeric antibody” is a recombinant protein that contains the variable domains and complementarity determining regions derived from a rodent antibody, while the remainder of the antibody molecule is derived from a human antibody.

“Humanized antibodies” are recombinant proteins in which murine
20 complementarity determining regions of a monoclonal antibody have been transferred from heavy and light variable chains of the murine immunoglobulin into a human variable domain.

As used herein, a “therapeutic agent” is a molecule or atom that is conjugated to an antibody moiety to produce a conjugate that is useful for therapy.
25 Examples of therapeutic agents include drugs, toxins, immunomodulators, chelators, boron compounds, photoactive agents or dyes, and radioisotopes.

A “detectable label” is a molecule or atom that can be conjugated to an antibody moiety to produce a molecule useful for diagnosis. Examples of detectable labels include chelators, photoactive agents, radioisotopes, fluorescent agents,
30 paramagnetic ions, or other marker moieties.

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A [Nilsson *et al.*, *EMBO J.* 4:1075 (1985); Nilsson *et al.*, *Methods Enzymol.* 198:3 (1991)], glutathione S transferase [Smith and Johnson, *Gene* 67:31 (1988)], Glu-Glu affinity tag [Grussenmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7952 (1985)], substance P, FLAG peptide [Hopp *et al.*, *Biotechnology* 6:1204 (1988)], streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford *et al.*, *Protein Expression and Purification* 2:95 (1991). DNAs encoding affinity tags are available from commercial suppliers (*e.g.*, Pharmacia Biotech, Piscataway, NJ).

A "naked antibody" is an entire antibody, as opposed to an antibody fragment, which is not conjugated with a therapeutic agent. Naked antibodies include both polyclonal and monoclonal antibodies, as well as certain recombinant antibodies, such as chimeric and humanized antibodies.

As used herein, the term "antibody component" includes both an entire antibody and an antibody fragment.

An "immunoconjugate" is a conjugate of an antibody component with a therapeutic agent or a detectable label.

As used herein, the term "antibody fusion protein" refers to a recombinant molecule that comprises an antibody component and a therapeutic agent. Examples of therapeutic agents suitable for such fusion proteins include immunomodulators ("antibody-immunomodulator fusion protein") and toxins ("antibody-toxin fusion protein").

A "tumor associated antigen" is a protein normally not expressed, or expressed at lower levels, by a normal counterpart cell. Examples of tumor-associated antigens include alpha-fetoprotein, carcinoembryonic antigen, and Her-2/neu. Many other illustrations of tumor-associated antigens are known to those of skill in the art. See, for example, Urban *et al.*, *Ann. Rev. Immunol.* 10:617 (1992).

T062180-4774660

As used herein, an "infectious agent" denotes both microbes and parasites. A "microbe" includes viruses, bacteria, rickettsia, mycoplasma, protozoa, fungi and like microorganisms. A "parasite" denotes infectious, generally microscopic or very small multicellular invertebrates, or ova or juvenile forms thereof, which are 5 susceptible to immune-mediated clearance or lytic or phagocytic destruction, such as malarial parasites, spirochetes, and the like.

An "infectious agent antigen" is an antigen associated with an infectious agent.

A "target polypeptide" or a "target peptide" is an amino acid sequence 10 that comprises at least one epitope, and that is expressed on a target cell, such as a tumor cell, or a cell that carries an infectious agent antigen. T cells recognize peptide epitopes presented by a major histocompatibility complex molecule to a target polypeptide or target peptide and typically lyse the target cell or recruit other immune cells to the site of the target cell, thereby killing the target cell.

15 An "antigenic peptide" is a peptide that will bind a major histocompatibility complex molecule to form an MHC-peptide complex, which is recognized by a T cell, thereby inducing a cytotoxic lymphocyte response upon presentation to the T cell. Thus, antigenic peptides are capable of binding to an appropriate major histocompatibility complex molecule and inducing a cytotoxic T 20 cells response, such as cell lysis or specific cytokine release against the target cell that binds or expresses the antigen. The antigenic peptide can be bound in the context of a class I or class II major histocompatibility complex molecule, on an antigen presenting cell or on a target cell.

In eukaryotes, RNA polymerase II catalyzes the transcription of a 25 structural gene to produce mRNA. A nucleic acid molecule can be designed to contain an RNA polymerase II template in which the RNA transcript has a sequence that is complementary to that of a specific mRNA. The RNA transcript is termed an "anti-sense RNA" and a nucleic acid molecule that encodes the anti-sense RNA is termed an "anti-sense gene." Anti-sense RNA molecules are capable of binding to mRNA 30 molecules, resulting in an inhibition of mRNA translation.

DRAFT EDITION 2020

An “anti-sense oligonucleotide specific for Zcys8” or a “Zcys8 anti-sense oligonucleotide” is an oligonucleotide having a sequence (a) capable of forming a stable triplex with a portion of the Zcys8 gene, or (b) capable of forming a stable duplex with a portion of an mRNA transcript of the Zcys8 gene.

5 A “ribozyme” is a nucleic acid molecule that contains a catalytic center. The term includes RNA enzymes, self-splicing RNAs, self-cleaving RNAs, and nucleic acid molecules that perform these catalytic functions. A nucleic acid molecule that encodes a ribozyme is termed a “ribozyme gene.”

10 An “external guide sequence” is a nucleic acid molecule that directs the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, resulting in the cleavage of the mRNA by RNase P. A nucleic acid molecule that encodes an external guide sequence is termed an “external guide sequence gene.”

15 The term “variant human Zcys8 gene” refers to nucleic acid molecules that encode a polypeptide having an amino acid sequence that is a modification of SEQ ID NO: 2. Such variants include naturally-occurring polymorphisms of Zcys8 genes, as well as synthetic genes that contain conservative amino acid substitutions of the amino acid sequence of SEQ ID NO: 2. Additional variant forms of Zcys8 genes are nucleic acid molecules that contain insertions or deletions of the nucleotide sequences described herein. A variant Zcys8 gene can be identified by determining whether the 20 gene hybridizes with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 1, or its complement, under stringent conditions.

25 Alternatively, variant Zcys8 genes can be identified by sequence comparison. Two amino acid sequences have “100% amino acid sequence identity” if the amino acid residues of the two amino acid sequences are the same when aligned for maximal correspondence. Similarly, two nucleotide sequences have “100% nucleotide sequence identity” if the nucleotide residues of the two nucleotide sequences are the same when aligned for maximal correspondence. Sequence comparisons can be performed using standard software programs such as those included in the LASERGENE bioinformatics computing suite, which is produced by DNASTAR 30 (Madison, Wisconsin). Other methods for comparing two nucleotide or amino acid sequences by determining optimal alignment are well-known to those of skill in the art

[see, for example, Peruski and Peruski, *The Internet and the New Biology: Tools for Genomic and Molecular Research* (ASM Press, Inc. 1997), Wu et al. (eds.), "Information Superhighway and Computer Databases of Nucleic Acids and Proteins," in *Methods in Gene Biotechnology*, pages 123-151 (CRC Press, Inc. 1997), and Bishop 5 (ed.), *Guide to Human Genome Computing*, 2nd Edition (Academic Press, Inc. 1998)]. Particular methods for determining sequence identity are described below.

Regardless of the particular method used to identify a variant Zcys8gene or variant Zcys8 polypeptide, a variant gene or polypeptide encoded by a variant gene is functionally characterized by either its ability to bind specifically to an anti-Zcys8 10 antibody.

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or 15 may encode polypeptides having altered amino acid sequence. The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

"Paralogs" are distinct but structurally related proteins made by an 20 organism. Paralogs are believed to arise through gene duplication. For example, α -globin, β -globin, and myoglobin are paralogs of each other.

The present invention includes functional fragments of Zcys8genes. Within the context of this invention, a "functional fragment" of a Zcys8 gene refers to a 25 nucleic acid molecule that encodes a portion of a Zcys8 polypeptide which either (1) possesses an anti-viral or anti-proliferative activity, or (2) specifically binds with an anti-Zcys8 antibody. For example, a functional fragment of a human Zcys8 gene described herein comprises a portion of the nucleotide sequence of SEQ ID NO:1. Due to the imprecision of standard analytical methods, molecular weights and lengths of polymers are understood to be approximate values. When such a value is expressed as 30 "about" X or "approximately" X, the stated value of X will be understood to be accurate to $\pm 10\%$.

3. Production of the Human Zcys8 Gene

Nucleic acid molecules encoding a human Zcys8gene can be obtained by screening a human brain cDNA or genomic library using polynucleotide probes based upon SEQ ID NO: 1. These techniques are standard and well established.

As an illustration, a nucleic acid molecule that encodes a human Zcys8gene can be isolated from a human cDNA library. In this case, the first step would be to prepare the cDNA library by isolating RNA from testis or colon tissue, using methods well-known to those of skill in the art. In general, RNA isolation techniques must provide a method for breaking cells, a means of inhibiting RNase-directed degradation of RNA, and a method of separating RNA from DNA, protein, and polysaccharide contaminants. For example, total RNA can be isolated by freezing tissue in liquid nitrogen, grinding the frozen tissue with a mortar and pestle to lyse the cells, extracting the ground tissue with a solution of phenol/chloroform to remove proteins, and separating RNA from the remaining impurities by selective precipitation with lithium chloride (see, for example, Ausubel *et al.* (eds.), *Short Protocols in Molecular Biology, 3rd Edition*, pages 4-1 to 4-6 (John Wiley & Sons 1995) [“Ausubel (1995)”]; Wu *et al.*, *Methods in Gene Biotechnology*, pages 33-41 (CRC Press, Inc. 1997)).

Alternatively, total RNA can be isolated from brain by extracting ground tissue with guanidinium isothiocyanate, extracting with organic solvents, and separating RNA from contaminants using differential centrifugation (see, for example, Chirgwin *et al.*, *Biochemistry* 18:52 (1979); Ausubel (1995) at pages 4-1 to 4-6; Wu (1997) at pages 33-41).

To construct a cDNA library, poly(A)⁺ RNA must be isolated from a total RNA preparation. Poly(A)⁺ RNA can be isolated from total RNA using the standard technique of oligo(dT)-cellulose chromatography (see, for example, Aviv and Leder, *Proc. Nat'l Acad. Sci. USA* 69:1408 (1972); Ausubel (1995) at pages 4-11 to 4-12).

Double-stranded cDNA molecules are synthesized from poly(A)⁺ RNA using techniques well-known to those in the art. (See, for example, Wu (1997) at pages 41-46). Moreover, commercially available kits can be used to synthesize double-stranded cDNA molecules. For example, such kits are available from Life

Technologies, Inc. (Gaithersburg, MD), CLONTECH Laboratories, Inc. (Palo Alto, CA), Promega Corporation (Madison, WI) and STRATAGENE (La Jolla, CA).

- Various cloning vectors are appropriate for the construction of a cDNA library. For example, a cDNA library can be prepared in a vector derived from 5 bacteriophage, such as a λgt10 vector. See, for example, Huynh *et al.*, "Constructing and Screening cDNA Libraries in λgt10 and λgt11," in *DNA Cloning: A Practical Approach Vol. I*, Glover (ed.), page 49 (IRL Press, 1985); Wu (1997) at pages 47-52.

- Alternatively, double-stranded cDNA molecules can be inserted into a plasmid vector, such as a PBLUESCRIPT vector (STRATAGENE; La Jolla, CA), a 10 LAMDAGEM-4 (Promega Corp.) or other commercially available vectors. Suitable cloning vectors also can be obtained from the American Type Culture Collection (Manassas, VA).

- To amplify the cloned cDNA molecules, the cDNA library is inserted into a prokaryotic host, using standard techniques. For example, a cDNA library can be 15 introduced into competent *E. coli* DH5 cells, which can be obtained, for example, from Life Technologies, Inc. (Gaithersburg, MD).

- A human genomic library can be prepared by means well known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327). Genomic DNA can be isolated by lysing tissue with the detergent Sarkosyl, digesting the 20 lysate with proteinase K, clearing insoluble debris from the lysate by centrifugation, precipitating nucleic acid from the lysate using isopropanol, and purifying resuspended DNA on a cesium chloride density gradient.

- DNA fragments that are suitable for the production of a genomic library can be obtained by the random shearing of genomic DNA or by the partial digestion of 25 genomic DNA with restriction endonucleases. Genomic DNA fragments can be inserted into a vector, such as a bacteriophage or cosmid vector, in accordance with conventional techniques, such as the use of restriction enzyme digestion to provide appropriate termini, the use of alkaline phosphatase treatment to avoid undesirable joining of DNA molecules, and ligation with appropriate ligases. Techniques for such manipulation are well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327).

Nucleic acid molecules that encode a human Zcys8 gene can also be obtained using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon the nucleotide sequences of the human Zcys8 gene, as described herein. General methods for screening libraries with PCR are provided by, for example, Yu *et al.*, "Use of the Polymerase Chain Reaction to Screen Phage Libraries," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 211-215 (Humana Press, Inc. 1993). Moreover, techniques for using PCR to isolate related genes are described by, for example, Preston, "Use of Degenerate Oligonucleotide Primers and the Polymerase Chain Reaction to Clone Gene Family Members," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 317-337 (Humana Press, Inc. 1993). Alternatively, human genomic libraries can be obtained from commercial sources such as Research Genetics (Huntsville, AL) and the American Type Culture Collection (Manassas, VA).

A library containing cDNA or genomic clones can be screened with one or more polynucleotide probes based upon SEQ ID NO: 1, using standard methods (see, for example, Ausubel (1995) at pages 6-1 to 6-11).

Anti-Zcys8 antibodies, produced as described below, can also be used to isolate DNA sequences that encode human Zcys8 genes from cDNA libraries. For example, the antibodies can be used to screen λgt11 expression libraries, or the antibodies can be used for immunoscreening following hybrid selection and translation [see, for example, Ausubel (1995) at pages 6-12 to 6-16; Margolis *et al.*, "Screening λ expression libraries with antibody and protein probes," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 1-14 (Oxford University Press 1995)].

As an alternative, a Zcys8 gene can be obtained by synthesizing nucleic acid molecules using mutually priming long oligonucleotides and the nucleotide sequences described herein (see, for example, Ausubel (1995) at pages 8-8 to 8-9). Established techniques using the polymerase chain reaction provide the ability to synthesize DNA molecules at least two kilobases in length [Adang *et al.*, *Plant Molec. Biol.* 21:1131 (1993), Bambot *et al.*, *PCR Methods and Applications* 2:266 (1993), Dillon *et al.*, "Use of the Polymerase Chain Reaction for the Rapid Construction of

Synthetic Genes," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 263-268, (Humana Press, Inc. 1993), and Holowachuk *et al.*, *PCR Methods Appl.* 4:299 (1995)].

The nucleic acid molecules of the present invention can also be
5 synthesized with "DNA synthesizers" using protocols such as the phosphoramidite method. If chemically synthesized double stranded DNA is required for an application such as the synthesis of a gene or a gene fragment, then each complementary strand is made separately. The production of short genes (60 to 80 base pairs) is technically straightforward and can be accomplished by synthesizing the complementary strands
10 and then annealing them. For the production of longer genes (>300 base pairs), however, special strategies may be required, because the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length.

15 One method for building a synthetic gene requires the initial production of a set of overlapping, complementary oligonucleotides, each of which is 20 to 60 nucleotides long. The sequences of the strands are planned so that, after annealing, the two end segments of the gene are aligned to give blunt ends. Each internal section of the gene has complementary 3' and 5' terminal extensions that are designed to base pair
20 precisely with an adjacent section. Thus, after the gene is assembled, the only remaining requirement to complete the process is to seal the nicks along the backbones of the two strands with T4 DNA ligase. In addition to the protein coding sequence, synthetic genes can be designed with terminal sequences that facilitate insertion into a restriction
endonuclease sites of a cloning vector and other sequences should also be added that
25 contain signals for the proper initiation and termination of transcription and translation.

An alternative way to prepare a full-size gene is to synthesize a specified set of overlapping oligonucleotides (40 to 100 nucleotides). After the 3' and 5' extensions (6 to 10 nucleotides) are annealed, large gaps still remain, but the base-paired regions are both long enough and stable enough to hold the structure together.
30 The duplex is completed and the gaps filled by enzymatic DNA synthesis with *E. coli* DNA polymerase I. This enzyme uses the 3'-hydroxyl groups as replication initiation

TOE9001650

points and the single-stranded regions as templates. After the enzymatic synthesis is completed, the nicks are sealed with T4 DNA ligase. For larger genes, the complete gene sequence is usually assembled from double-stranded fragments that are each put together by joining four to six overlapping oligonucleotides (20 to 60 base pairs each).

- 5 If there is a sufficient amount of the double-stranded fragments after each synthesis and annealing step, they are simply joined to one another. Otherwise, each fragment is cloned into a vector to amplify the amount of DNA available. In both cases, the double-stranded constructs are sequentially linked to one another to form the entire gene sequence. Each double-stranded fragment and the complete sequence should be
- 10 characterized by DNA sequence analysis to verify that the chemically synthesized gene has the correct nucleotide sequence. For reviews on polynucleotide synthesis, see, for example, Glick and Pasternak, *Molecular Biotechnology, Principles and Applications of Recombinant DNA* (ASM Press 1994), Itakura *et al.*, *Annu. Rev. Biochem.* 53:323 (1984), and Climie *et al.*, *Proc. Nat'l Acad. Sci. USA* 87:633 (1990).

15 The sequence of a *Zcys8*cDNA or *Zcys8* genomic fragment can be determined using standard methods. Moreover, the identification of genomic fragments containing a *Zcys8* promoter or regulatory element can be achieved using well-established techniques, such as deletion analysis (see, generally, Ausubel (1995)).

Cloning of 5' flanking sequences also facilitates production of *Zcys8* proteins by "gene activation," following the methods disclosed in U.S. Patent No. 5,641,670. Briefly, expression of an endogenous *Zcys8*gene in a cell is altered by introducing into the *Zcys8*locus a DNA construct comprising at least a targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site. The targeting sequence is a *Zcys8* 5' non-coding sequence that permits homologous recombination of the construct with the endogenous *Zcys8* locus, whereby the sequences within the construct become operably linked with the endogenous *Zcys8* coding sequence. In this way, an endogenous *Zcys8*promoter can be replaced or supplemented with other regulatory sequences to provide enhanced, tissue-specific, or otherwise regulated expression.

4. *Production of Zcys8 Gene Variants*

The present invention provides a variety of nucleic acid molecules, including DNA and RNA molecules that encode the Zcys8 polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these 5 polynucleotide molecules.

Table 1 sets forth the one-letter codes used to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, 10 the code Y denotes either C or T, and its complement R denotes A or G, A being complementary to T, and G being complementary to C.

T06280 - 4747460

Table 1

Nucleotide	Resolution	Complement	Resolution
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A G	Y	C T
Y	C T	R	A G
M	A C	K	G T
K	G T	M	A C
S	C G	S	C G
W	A T	W	A T
H	A C T	D	A G T
B	C G T	V	A C G
V	A C G	B	C G T
D	A G T	H	A C T
N	A C G T	N	A C G T

The degenerate codons encompassing all possible codons for a given amino acid, are set forth in Table 2.

Table 2

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn Asp	B		RAY
Glu Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding an amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO: 2. Variant sequences can be readily tested for functionality as described herein.

Different species can exhibit “preferential codon usage.” In general, see, Grantham *et al.*, *Nuc. Acids Res.* 8:1893 (1980), Haas *et al.* *Curr. Biol.* 6:315 (1996), Wain-Hobson *et al.*, *Gene* 13:355 (1981), Grosjean and Fiers, *Gene* 18:199 (1982), Holm, *Nuc. Acids Res.* 14:3075 (1986), Ikemura, *J. Mol. Biol.* 158:573 (1982), Sharp and Matassi, *Curr. Opin. Genet. Dev.* 4:851 (1994), Kane, *Curr. Opin. Biotechnol.* 6:494 (1995), and Makrides, *Microbiol. Rev.* 60:512 (1996). As used herein, the term “preferential codon usage” or “preferential codons” is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid (See Table 2). For example, the amino acid Threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Therefore, a degenerate codon sequences can serve as templates for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein. Sequences containing preferential codons can be tested and optimized for expression in various species, and tested for functionality as disclosed herein.

The present invention further provides variant polypeptides and nucleic acid molecules that represent counterparts from other species (orthologs). These species include, but are not limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are Zcys8 5 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Orthologs of human Zcys8 can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses Zcys8 as 10 disclosed herein. Suitable sources of mRNA can be identified by probing northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line.

A Zcys8-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of 15 degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction with primers designed from the representative human Zcys8 sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to Zcys8 polypeptide. Similar techniques can also be 20 applied to the isolation of genomic clones, and to the isolation of nucleic molecules that encode murine Zcys8.

Those skilled in the art will recognize that the sequence disclosed in SEQ ID NO: 1 represents a single allele of human Zcys8, and that allelic variation and alternative splicing are expected to occur. Allelic variants of this sequence can be 25 cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the nucleotide sequence shown in SEQ ID NO: 1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NOs: 2 - 17. cDNA molecules generated 30 from alternatively spliced mRNAs, which retain the properties of the Zcys8 polypeptide are included within the scope of the present invention, as are polypeptides encoded by

such cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

Within preferred embodiments of the invention, isolated nucleic acid molecules that encode human Zcys8 can hybridize to nucleic acid molecules having the nucleotide sequence of SEQ ID NO: 1, or a sequence complementary thereto, under "stringent conditions." In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe.

As an illustration, a nucleic acid molecule encoding a variant Zcys8 polypeptide can be hybridized with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 1 (or its complement) at 42°C overnight in a solution comprising 50% formamide, 5xSSC (1xSSC: 0.15 M sodium chloride and 15 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution (100x Denhardt's solution: 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone, and 2% (w/v) bovine serum albumin), 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA. One of skill in the art can devise variations of these hybridization conditions. For example, the hybridization mixture can be incubated at a higher temperature, such as about 65°C, in a solution that does not contain formamide. Moreover, premixed hybridization solutions are available (e.g., EXPRESSHYB Hybridization Solution from CLONTECH Laboratories, Inc.), and hybridization can be performed according to the manufacturer's instructions.

Following hybridization, the nucleic acid molecules can be washed to remove non-hybridized nucleic acid molecules under stringent conditions, or under highly stringent conditions. Typical stringent washing conditions include washing in a solution of 0.5x - 2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 55 - 65°C. That is, nucleic acid molecules encoding a variant Zcys8 polypeptide hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under stringent washing conditions, in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, including 0.5x SSC with

DOCUMENT NUMBER

0.1% SDS at 55°C, or 2xSSC with 0.1% SDS at 65°C. One of skill in the art can readily devise equivalent conditions, for example, by substituting SSPE for SSC in the wash solution.

- Typical highly stringent washing conditions include washing in a
- 5 solution of 0.1x - 0.2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 50 - 65°C. In other words, nucleic acid molecules encoding a variant Zcys8 polypeptide hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under highly stringent washing conditions, in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 - 65°C, including 0.1x SSC with
- 10 0.1% SDS at 50°C, or 0.2xSSC with 0.1% SDS at 65°C.

The present invention also provides isolated Zcys8 polypeptides that have a substantially similar sequence identity to the polypeptides of SEQ ID NOs: 2, 3, 4 or their orthologs. The term “substantially similar sequence identity” is used herein to denote polypeptides having at least 70%, at least 80%, at least 90%, at least 95% or 15 greater than 95% sequence identity to the sequence shown in SEQ ID NO: 2 or their orthologs.

The present invention also contemplates Zcys8 variant nucleic acid molecules that can be identified using two criteria: a determination of the similarity between the encoded polypeptide with the amino acid sequence of SEQ ID NO: 2, and a 20 hybridization assay, as described above. Such Zcys8 variants include nucleic acid molecules (1) that hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 1 (or its complement) under stringent washing conditions, in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, and (2) that encode a polypeptide having at least 70%, at least 80%, at least 90%, at 25 least 95% or greater than 95% sequence identity to the amino acid sequence of SEQ ID NO: 2. Alternatively, Zcys8 variants can be characterized as nucleic acid molecules (1) that hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 1 (or its complement) under highly stringent washing conditions, in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 - 65°C, and (2) 30 that encode a polypeptide having at least 70%, at least 80%, at least 90%, at least 95% or greater than 95% sequence identity to the amino acid sequence of SEQ ID NO: 2.

DRAFT EDITION 2010 FORM

Percent sequence identity is determined by conventional methods. See, for example, Altschul *et al.*, *Bull. Math. Bio.* 48:603 (1986), and Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM 62" scoring matrix of Henikoff and Henikoff (*ibid.*) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as: ([Total number of identical matches]/ [length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences])(100).

TOP SECRET - EYES ONLY

Table 3

A R N D C Q E G H I L K M F P S T W Y V

A 4

R -1 5

N -2 0 6

D -2 -2 1 6

C 0 -3 -3 -3 9

Q -1 1 0 0 -3 5

E -1 0 0 2 -4 2 5

G 0 -2 0 -1 -3 -2 -2 6

H -2 0 1 -1 -3 0 0 -2 8

I -1 -3 -3 -3 -1 -3 -3 -4 -3 4

L -1 -2 -3 -4 -1 -2 -3 -4 -3 2 4

K -1 2 0 -1 -3 1 1 -2 -1 -3 -2 5

M -1 -1 -2 -3 -1 0 -2 -3 -2 1 2 -1 5

F -2 -3 -3 -2 -3 -3 -3 -1 0 0 -3 0 6

P -1 -2 -2 -1 -3 -1 -1 -2 -2 -3 -3 -1 -2 -4 7

S 1 -1 1 0 -1 0 0 0 -1 -2 -2 0 -1 -2 -1 4

T 0 -1 0 -1 -1 -1 -2 -2 -1 -1 -1 -2 -1 1 5

W -3 -3 -4 -4 -2 -2 -3 -2 -2 -3 -2 -3 -1 1 -4 -3 -2 11

Y -2 -2 -2 -3 -2 -1 -2 -3 2 -1 -1 -2 -1 3 -3 -2 -2 2 7

V 0 -3 -3 -3 -1 -2 -2 -3 -3 3 1 -2 1 -1 -2 -2 0 -3 -1 4

5

10

15

20

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative Zcys8 variant. The FASTA algorithm is described by Pearson and Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444 (1988), and by Pearson, *Meth. Enzymol.* 183:63 (1990). Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (e.g., SEQ ID NO:2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then re-scored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, *J. Mol. Biol.* 48:444 (1970); Sellers, *SIAM J. Appl. Math.* 26:787 (1974)), which allows for amino acid insertions and deletions. Illustrative parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol.* 183:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, most preferably three, with other parameters set as described above.

The present invention includes nucleic acid molecules that encode a polypeptide having a conservative amino acid change, compared with the amino acid

T06280-11ET4650

sequence of SEQ ID NO: 2 . That is, variants can be obtained that contain one or more amino acid substitutions of SEQ ID NO: 2, in which an alkyl amino acid is substituted for an alkyl amino acid in an Zcys8 amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in an Zcys8 amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in an Zcys8 amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-containing amino acid in an Zcys8 amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in an Zcys8 amino acid sequence, a basic amino acid is substituted for a basic amino acid in an Zcys8 amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in an Zcys8 amino acid sequence.

Among the common amino acids, for example, a “conservative amino acid substitution” is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine. Additional variants can be obtained by producing polypeptides having two or more of these amino acid substitutions.

The BLOSUM62 table is an amino acid substitution matrix derived from about 2,000 local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins (Henikoff and Henikoff, *Proc. Nat'l Acad. Sci. USA* 89:10915 (1992)). Accordingly, the BLOSUM62 substitution frequencies can be used to define conservative amino acid substitutions that may be introduced into the amino acid sequences of the present invention. Although it is possible to design amino acid substitutions based solely upon chemical properties (as discussed above), the language “conservative amino acid substitution” preferably refers to a substitution represented by a BLOSUM62 value of greater than -1. For example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0, 1, 2, or 3. According to this system, preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (*e.g.*, 1,

2 or 3), while more preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 2 (e.g., 2 or 3).

Conservative amino acid changes in an *Zcys8* gene can be introduced by substituting nucleotides for the nucleotides recited in SEQ ID NO: 1. Such

5 "conservative amino acid" variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like (see Ausubel (1995) at pages 8-10 to 8-22; and McPherson (ed.), *Directed Mutagenesis: A Practical Approach* (IRL Press 1991)). The ability of such variants to be immunogenic so that they can be used in the production of

10 antibodies that can be used to isolate or purify native *Zcys8* can be determined using routine assays described below. A variant *Zcys8* polypeptide can be identified by the ability to specifically bind anti-*Zcys8* antibodies.

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is typically carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson *et al.*, *J. Am. Chem. Soc.* 113:2722 (1991), Ellman *et al.*, *Methods Enzymol.* 202:301 (1991), Chung *et al.*, *Science* 259:806 (1993), and Chung *et al.*, *Proc. Nat'l Acad. Sci. USA* 90:10145 (1993).

DRAFT - PROVISIONAL

In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs [Turcatti *et al.*, *J. Biol. Chem.* 271:19991 (1996)]. Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (*e.g.*, phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (*e.g.*, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide *et al.*, *Biochem.* 33:7470 (1994). Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions [Wynn and Richards, *Protein Sci.* 2:395 (1993)].

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for Zcys8 amino acid residues.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081 (1989), Bass *et al.*, *Proc. Nat'l Acad. Sci. USA* 88:4498 (1991), Coombs and Corey, "Site-Directed Mutagenesis and Protein Engineering," in *Proteins: Analysis and Design*, Angeletti (ed.), pages 259-311 (Academic Press, Inc. 1998)). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, *J. Biol. Chem.* 271:4699 (1996) Although sequence analysis can be used to identify Zcys8 receptor binding sites, the location of Zcys8 receptor binding domains can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, *Science* 255:306 (1992), Smith *et al.*, *J. Mol.*

DRAFT EDITION 10/2000

Biol. 224:899 (1992), and Wlodaver *et al.*, *FEBS Lett.* 309:59 (1992). Moreover, Zcys8 labeled with biotin or FITC can be used for expression cloning of Zcys8 receptors.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer [*Science* 241:53 (1988)] or Bowie and Sauer [*Proc. Nat'l Acad. Sci. USA* 86:2152 (1989)]. Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display [e.g., Lowman *et al.*, *Biochem.* 30:10832 (1991), Ladner *et al.*, U.S. Patent No. 5,223,409, Huse, international publication No. WO 92/06204, and region-directed mutagenesis (Derbyshire *et al.*, *Gene* 46:145 (1986), and Ner *et al.*, *DNA* 7:127, (1988)].

Variants of the disclosed Zcys8nucleotide and polypeptide sequences can also be generated through DNA shuffling as disclosed by Stemmer, *Nature* 370:389 (1994), Stemmer, *Proc. Nat'l Acad. Sci. USA* 91:10747 (1994), and international publication No. WO 97/20078. Briefly, variant DNAs are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNAs, such as allelic variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid “evolution” of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed herein can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode biologically active polypeptides, or polypeptides that bind with anti-Zcys8 antibodies, can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

The present invention also includes "functional fragments" of Zcys8 polypeptides and nucleic acid molecules encoding such functional fragments. Routine deletion analyses of nucleic acid molecules can be performed to obtain functional fragments of a nucleic acid molecule that encodes a Zcys8 polypeptide. As an illustration, DNA molecules having the nucleotide sequence of SEQ ID NO: 1 can be digested with *Bal*31 nuclease to obtain a series of nested deletions. The fragments are then inserted into expression vectors in proper reading frame, and the expressed polypeptides are isolated and tested for the ability to bind anti-Zcys8 antibodies. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired fragment. Alternatively, particular fragments of a Zcys8 gene can be synthesized using the polymerase chain reaction.

The present invention also contemplates functional fragments of an Zcys8 gene that has amino acid changes, compared with the amino acid sequence of SEQ ID NO: 2. An alternative approach to identifying a variant gene on the basis of structure is to determine whether a nucleic acid molecule encoding a potential variant Zcys8 gene can hybridize to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 1 as discussed above.

The present invention also provides polypeptide fragments or peptides comprising an epitope-bearing portion of a Zcys8 polypeptide described herein. Such fragments or peptides may comprise an "immunogenic epitope," which is a part of a protein that elicits an antibody response when the entire protein is used as an immunogen. Immunogenic epitope-bearing peptides can be identified using standard methods (see, for example, Geysen *et al.*, *Proc. Nat'l Acad. Sci. USA* 81:3998 (1983)).

In contrast, polypeptide fragments or peptides may comprise an "antigenic epitope," which is a region of a protein molecule to which an antibody can specifically bind. Certain epitopes consist of a linear or contiguous stretch of amino acids, and the antigenicity of such an epitope is not disrupted by denaturing agents. It is known in the art that relatively short synthetic peptides that can mimic epitopes of a protein can be used to stimulate the production of antibodies against the protein [see, for example, Sutcliffe *et al.*, *Science* 219:660 (1983)]. Accordingly, antigenic epitope-

bearing peptides and polypeptides of the present invention are useful to raise antibodies that bind with the polypeptides described herein.

Antigenic epitope-bearing peptides and polypeptides preferably contain at least four to ten amino acids, at least ten to fifteen amino acids, or about 15 to about 30 amino acids of SEQ ID NO: 2. Such epitope-bearing peptides and polypeptides can be produced by fragmenting a Zcys8 polypeptide, or by chemical peptide synthesis, as described herein. Moreover, epitopes can be selected by phage display of random peptide libraries [see, for example, Lane and Stephen, *Curr. Opin. Immunol.* 5:268 (1993), and Cortese *et al.*, *Curr. Opin. Biotechnol.* 7:616 (1996)]. Standard methods for identifying epitopes and producing antibodies from small peptides that comprise an epitope are described, for example, by Mole, "Epitope Mapping," in *Methods in Molecular Biology*, Vol. 10, Manson (ed.), pages 105-116 (The Humana Press, Inc. 1992), Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in *Monoclonal Antibodies: Production, Engineering, and Clinical Application*, Ritter and Ladyman (eds.), pages 60-84 (Cambridge University Press 1995), and Coligan *et al.* (eds.), *Current Protocols in Immunology*, pages 9.3.1 - 9.3.5 and pages 9.4.1 - 9.4.11 (John Wiley & Sons 1997).

Regardless of the particular nucleotide sequence of a variant Zcys8gene, the gene encodes a polypeptide that is characterized by its ability to bind specifically to an anti-Zcys8 antibody. More specifically, variant human Zcys8genes encode polypeptides that exhibit at least 50%, and preferably, greater than 70, 80, or 90%, of the activity of polypeptide encoded by the human Zcys8gene described herein.

For any Zcys8 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 1 and 2 above. Moreover, those of skill in the art can use standard software to devise Zcys8 variants based upon the nucleotide and amino acid sequences described herein. Accordingly, the present invention includes a computer-readable medium encoded with a data structure that provides at least one of the following sequences: SEQ ID NOs: 1 -18. For example, a computer-readable medium can be encoded with a data structure that provides at least one of the following sequences: SEQ ID NO:1, SEQ ID NOs:2-18.

SEARCHED
INDEXED
COPIED
FILED

Suitable forms of computer-readable media include magnetic media and optically readable media. Examples of magnetic media include a hard or fixed drive, a random access memory (RAM) chip, a floppy disk, digital linear tape (DLT), a disk cache, and a ZIP disk. Optically readable media are exemplified by compact discs (*e.g.*, CD-read only memory (ROM), CD-re-writable (RW), and CD-recordable), and digital versatile/video discs (DVD) (*e.g.*, DVD-ROM, DVD-RAM, and DVD+RW).

5. ***Production of Zcys8 Fusion Proteins and Conjugates***

Fusion proteins of Zcys8 can be used to express Zcys8 in a recombinant host, and to isolate expressed Zcys8. As described below, particular Zcys8 fusion proteins also have uses in diagnosis and therapy.

One type of fusion protein comprises a peptide that guides a Zcys8 polypeptide from a recombinant host cell. To direct a Zcys8 polypeptide into the secretory pathway of a eukaryotic host cell, a secretory signal sequence (also known as a signal peptide, a leader sequence, prepro sequence or pre sequence) is provided in the Zcys8 expression vector. While the secretory signal sequence may be derived from Zcys8, a suitable signal sequence may also be derived from another secreted protein or synthesized *de novo*. The secretory signal sequence is operably linked to a Zcys8-encoding sequence such that the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleotide sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the nucleotide sequence of interest (see, *e.g.*, Welch *et al.*, U.S. Patent No. 5,037,743; Holland *et al.*, U.S. Patent No. 5,143,830).

25 Although the secretory signal sequence of Zcys8 or another protein produced by mammalian cells (*e.g.*, tissue-type plasminogen activator signal sequence, as described, for example, in U.S. Patent No. 5,641,655) is useful for expression of Zcys8 in recombinant mammalian hosts, a yeast signal sequence is preferred for expression in yeast cells. Examples of suitable yeast signal sequences are those derived from yeast mating pheromone α -factor (encoded by the *MF α 1* gene), invertase (encoded by the *SUC2* gene), or acid phosphatase (encoded by the *PHO5* gene). See, for

example, Romanos *et al.*, "Expression of Cloned Genes in Yeast," in *DNA Cloning 2: A Practical Approach*, 2nd Edition, Glover and Hames (eds.), pages 123-167 (Oxford University Press 1995).

In bacterial cells, it is often desirable to express a heterologous protein
5 as a fusion protein to decrease toxicity, increase stability, and to enhance recovery of
the expressed protein. For example, Zcys8 can be expressed as a fusion protein
comprising a glutathione S-transferase polypeptide. Glutathione S-transferase fusion
proteins are typically soluble, and easily purifiable from *E. coli* lysates on immobilized
glutathione columns. In similar approaches, a Zcys8 fusion protein comprising a
10 maltose binding protein polypeptide can be isolated with an amylose resin column,
while a fusion protein comprising the C-terminal end of a truncated Protein A gene can
be purified using IgG-Sepharose. Established techniques for expressing a heterologous
polypeptide as a fusion protein in a bacterial cell are described, for example, by
Williams *et al.*, "Expression of Foreign Proteins in *E. coli* Using Plasmid Vectors and
15 Purification of Specific Polyclonal Antibodies," in *DNA Cloning 2: A Practical
Approach*, 2nd Edition, Glover and Hames (Eds.), pages 15-58 (Oxford University Press
1995). In addition, commercially available expression systems are available. For
example, the PINPOINT Xa protein purification system (Promega Corporation;
20 Madison, WI) provides a method for isolating a fusion protein comprising a polypeptide
that becomes biotinylated during expression with a resin that comprises avidin.

Peptide tags that are useful for isolating heterologous polypeptides
expressed by either prokaryotic or eukaryotic cells include polyHistidine tags (which
have an affinity for nickel-chelating resin), *c-myc* tags, calmodulin binding protein
(isolated with calmodulin affinity chromatography), substance P, the RYIRS tag (which
25 binds with anti-RYIRS antibodies), the Glu-Glu tag, and the FLAG tag (which binds
with anti-FLAG antibodies). See, for example, Luo *et al.*, *Arch. Biochem. Biophys.*
329:215 (1996), Morganti *et al.*, *Biotechnol. Appl. Biochem.* 23:67 (1996), and Zheng
et al., *Gene* 186:55 (1997). Nucleic acid molecules encoding such peptide tags are
available, for example, from Sigma-Aldrich Corporation (St. Louis, MO).

The present invention also contemplates that the use of the secretory
30 signal sequence contained in the Zcys8 polypeptides of the present invention to direct

other polypeptides into the secretory pathway. A signal fusion polypeptide can be made wherein a secretory signal sequence derived from amino acid residues 1 to 28 of SEQ ID NO: 2 is operably linked to another polypeptide using methods known in the art and disclosed herein. The secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Such constructs have numerous applications known in the art. For example, these novel secretory signal sequence fusion constructs can direct the secretion of an active component of a normally non-secreted protein, such as a receptor. Such fusions may be used in a transgenic animal or in a cultured recombinant host to direct peptides through the secretory pathway. With regard to the latter, exemplary polypeptides include pharmaceutically active molecules such as Factor VIIa, proinsulin, insulin, follicle stimulating hormone, tissue type plasminogen activator, tumor necrosis factor, interleukins [*e.g.*, interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, and IL-15], colony stimulating factors (*e.g.*, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF)], interferons (*e.g.*, interferons- α , - β , - γ , - ω , - δ , and - τ), the stem cell growth factor designated “S1 factor,” erythropoietin, and thrombopoietin. The Zcys8 secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Fusion proteins comprising a Zcys8 secretory signal sequence can be constructed using standard techniques.

Another form of fusion protein comprises a Zcys8 polypeptide and an immunoglobulin heavy chain constant region, typically an F_C fragment, which contains two or three constant region domains and a hinge region but lacks the variable region. As an illustration, Chang *et al.*, U.S. Patent No. 5,723,125, describe a fusion protein comprising a human interferon and a human immunoglobulin Fc fragment. The C-terminal of the interferon is linked to the N-terminal of the Fc fragment by a peptide linker moiety. An example of a peptide linker is a peptide comprising primarily a T cell inert sequence, which is immunologically inert. An exemplary peptide linker has the amino acid sequence: GGSGG SGGGG SG S (SEQ ID NO: 19). In this fusion

TO BE MADE PUBLIC

protein, a preferred Fc moiety is a human $\gamma 4$ chain, which is stable in solution and has little or no complement activating activity. Accordingly, the present invention contemplates a Zcys8 fusion protein that comprises a Zcys8 moiety and a human Fc fragment, wherein the C-terminus of the Zcys8 moiety is attached to the N-terminus of the Fc fragment via a peptide linker. The Zcys8 moiety can be a Zcys8 molecule or a fragment thereof.

In another variation, an Zcys8 fusion protein comprises an IgG sequence, an Zcys8 moiety covalently joined to the aminoterminal end of the IgG sequence, and a signal peptide that is covalently joined to the aminoterminal of the Zcys8 moiety, wherein the IgG sequence consists of the following elements in the following order: a hinge region, a CH_2 domain, and a CH_3 domain. Accordingly, the IgG sequence lacks a CH_1 domain. The Zcys8 moiety displays a Zcys8 activity, as described herein, such as the ability to bind with a Zcys8 receptor. This general approach to producing fusion proteins that comprise both antibody and nonantibody portions has been described by LaRochelle *et al.*, EP 742830 (WO 95/21258).

Fusion proteins comprising a Zcys8 moiety and an Fc moiety can be used, for example, as an *in vitro* assay tool. For example, the presence of a Zcys8 receptor in a biological sample can be detected using a Zcys8-immunoglobulin fusion protein, in which the Zcys8 moiety is used to target the cognate receptor, and a macromolecule, such as Protein A or anti-Fc antibody, is used to detect the bound fusion protein-receptor complex. Moreover, such fusion proteins can be used to identify agonists and antagonists that interfere with the binding of Zcys8 to its receptor.

Similarly, fusion proteins can be constructed that comprise a murine Zcys8 polypeptide and an immunoglobulin heavy chain constant region.

In addition, antibody-Zcys8 fusion proteins, comprising antibody variable domains, are useful as therapeutic proteins, in which the antibody moiety binds with a target antigen, such as a tumor associated antigen. Methods of making antibody-cytokine fusion proteins are known to those of skill in the art. For example, antibody fusion proteins comprising an interleukin-2 moiety are described by Boleti *et al.*, *Ann. Oncol.* 6:945 (1995), Nicolet *et al.*, *Cancer Gene Ther.* 2:161 (1995), Becker *et al.*, *Proc. Nat'l Acad. Sci. USA* 93:7826 (1996), Hank *et al.*, *Clin. Cancer Res.* 2:1951 (1996), and Hu *et*

al., *Cancer Res.* 56:4998 (1996). Moreover, Yang *et al.*, *Hum. Antibodies Hybridomas* 6:129 (1995), and Xiang *et al.*, *J. Biotechnol.* 53:3 (1997), describe fusion proteins that include an F(ab')₂ fragment and a tumor necrosis factor alpha moiety. Additional cytokine-antibody fusion proteins include IL-8, IL-12, or Zcys8as the cytokine moiety (Holzer *et al.*, *Cytokine* 8:214 (1996); Gillies *et al.*, *J. Immunol.* 160:6195 (1998); Xiang *et al.*, *Hum. Antibodies Hybridomas* 7:2 (1996)). Also see, Gillies, U.S. Patent No. 5,650,150.

Moreover, using methods described in the art, hybrid Zcys8 proteins can be constructed using regions or domains of the inventive [see, for example, Picard, *Cur. Opin. Biology* 5:511 (1994)]. These methods allow the determination of the biological importance of larger domains or regions in a polypeptide of interest. Such hybrids may alter reaction kinetics, binding, constrict or expand the substrate specificity, or alter tissue and cellular localization of a polypeptide, and can be applied to polypeptides of unknown structure. Fusion proteins can be prepared by methods known to those skilled in the art by preparing each component of the fusion protein and chemically conjugating them. Alternatively, a polynucleotide encoding both components of the fusion protein in the proper reading frame can be generated using known techniques and expressed by the methods described herein. Moreover, such fusion proteins may exhibit other properties as disclosed herein. General methods for enzymatic and chemical cleavage of fusion proteins are described, for example, by Ausubel (1995) at pages 16-19 to 16-25.

The present invention also contemplates chemically modified Zcys8 compositions, in which a Zcys8 polypeptide is linked with a polymer. Typically, the polymer is water-soluble so that the Zcys8 conjugate does not precipitate in an aqueous environment, such as a physiological environment. An example of a suitable polymer is one that has been modified to have a single reactive group, such as an active ester for acylation, or an aldehyde for alkylation. In this way, the degree of polymerization can be controlled. An example of a reactive aldehyde is polyethylene glycol propionaldehyde, or mono-(C1-C10) alkoxy, or aryloxy derivatives thereof (see, for example, Harris, *et al.*, U.S. Patent No. 5,252,714). The polymer may be branched or

RECEIVED
MAY 16 1996
U.S. PATENT AND TRADEMARK OFFICE

unbranched. Moreover, a mixture of polymers can be used to produce Zcys8 conjugates.

Zcys8 conjugates used for therapy should preferably comprise pharmaceutically acceptable water-soluble polymer moieties. Conjugation of interferons with water-soluble polymers has been shown to enhance the circulating half-life of the interferon, and to reduce the immunogenicity of the polypeptide (see, for example, Nieforth *et al.*, *Clin. Pharmacol. Ther.* 59:636 (1996), and Monkash *et al.*, *Anal. Biochem.* 247:434 (1997)).

Suitable water-soluble polymers include polyethylene glycol (PEG), monomethoxy-PEG, mono-(C1-C10)alkoxy-PEG, aryloxy-PEG, poly-(N-vinyl pyrrolidone)PEG, tresyl monomethoxy PEG, PEG propionaldehyde, *bis*-succinimidyl carbonate PEG, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (*e.g.*, glycerol), polyvinyl alcohol, dextran, cellulose, or other carbohydrate-based polymers. Suitable PEG may have a molecular weight from about 600 to about 60,000, including, for example, 5,000, 12,000, 20,000 and 25,000. A Zcys8 conjugate can also comprise a mixture of such water-soluble polymers.

One example of a Zcys8 conjugate comprises a Zcys8 moiety and a polyalkyl oxide moiety attached to the *N*-terminus of the Zcys8 moiety. PEG is one suitable polyalkyl oxide. As an illustration, Zcys8 can be modified with PEG, a process known as "PEGylation." PEGylation of Zcys8 can be carried out by any of the PEGylation reactions known in the art (see, for example, EP 0 154 316, Delgado *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* 9:249 (1992), Duncan and Spreafico, *Clin. Pharmacokinet.* 27:290 (1994), and Francis *et al.*, *Int J Hematol* 68:1 (1998)). For example, PEGylation can be performed by an acylation reaction or by an alkylation reaction with a reactive polyethylene glycol molecule. In an alternative approach, Zcys8 conjugates are formed by condensing activated PEG, in which a terminal hydroxy or amino group of PEG has been replaced by an activated linker (see, for example, Karasiewicz *et al.*, U.S. Patent No. 5,382,657).

PEGylation by acylation typically requires reacting an active ester derivative of PEG with a Zcys8 polypeptide. An example of an activated PEG ester is

PEG esterified to *N*-hydroxysuccinimide. As used herein, the term "acylation" includes the following types of linkages between Zcys8 and a water-soluble polymer: amide, carbamate, urethane, and the like. Methods for preparing PEGylated Zcys8 by acylation will typically comprise the steps of (a) reacting an Zcys8 polypeptide with PEG (such as 5 a reactive ester of an aldehyde derivative of PEG) under conditions whereby one or more PEG groups attach to Zcys8, and (b) obtaining the reaction product(s). Generally, the optimal reaction conditions for acylation reactions will be determined based upon known parameters and desired results. For example, the larger the ratio of PEG: Zcys8, the greater the percentage of polyPEGylated Zcys8 product.

10 The product of PEGylation by acylation is typically a polyPEGylated Zcys8 product, wherein the lysine ϵ -amino groups are PEGylated via an acyl linking group. An example of a connecting linkage is an amide. Typically, the resulting Zcys8 will be at least 95% mono-, di-, or tri-pegylated, although some species with higher degrees of PEGylation may be formed depending upon the reaction conditions.

15 PEGylated species can be separated from unconjugated Zcys8 polypeptides using standard purification methods, such as dialysis, ultrafiltration, ion exchange chromatography, affinity chromatography, and the like.

20 PEGylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with Zcys8 in the presence of a reducing agent. PEG groups are preferably attached to the polypeptide via a -CH₂-NH group.

Derivatization via reductive alkylation to produce a monoPEGylated product takes advantage of the differential reactivity of different types of primary amino groups available for derivatization. Typically, the reaction is performed at a pH that allows one to take advantage of the pKa differences between the ϵ -amino groups of the 25 lysine residues and the α -amino group of the *N*-terminal residue of the protein. By such selective derivatization, attachment of a water-soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled. The conjugation with the polymer occurs predominantly at the *N*-terminus of the protein without significant modification of other reactive groups such as the lysine side chain amino groups. The present 30 invention provides a substantially homogenous preparation of Zcys8 monopolymer conjugates.

TOE280-C001

Reductive alkylation to produce a substantially homogenous population of monopolymer Zcys8 conjugate molecule can comprise the steps of: (a) reacting an Zcys8 polypeptide with a reactive PEG under reductive alkylation conditions at a pH suitable to permit selective modification of the α -amino group at the amino terminus of the Zcys8, and (b) obtaining the reaction product(s). The reducing agent used for reductive alkylation should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Preferred reducing agents include sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane, and pyridine borane.

For a substantially homogenous population of monopolymer Zcys8 conjugates, the reductive alkylation reaction conditions are those that permit the selective attachment of the water-soluble polymer moiety to the *N*-terminus of Zcys8. Such reaction conditions generally provide for pKa differences between the lysine amino groups and the α -amino group at the *N*-terminus. The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired because the less reactive the *N*-terminal α -group, the more polymer is needed to achieve optimal conditions. If the pH is higher, the polymer:Zcys8 need not be as large because more reactive groups are available. Typically, the pH will fall within the range of 3 - 9, or 3 - 6. Another factor to consider is the molecular weight of the water-soluble polymer. Generally, the higher the molecular weight of the polymer, the fewer number of polymer molecules which may be attached to the protein. For PEGylation reactions, the typical molecular weight is about 2 kDa to about 100 kDa, about 5 kDa to about 50 kDa, or about 12 kDa to about 25 kDa. The molar ratio of water-soluble polymer to Zcys8 will generally be in the range of 1:1 to 100:1. Typically, the molar ratio of water-soluble polymer to Zcys8 will be 1:1 to 20:1 for polyPEGylation, and 1:1 to 5:1 for monoPEGylation.

General methods for producing conjugates comprising interferon and water-soluble polymer moieties are known in the art. See, for example, Karasiewicz *et al.*, U.S. Patent No. 5,382,657, Greenwald *et al.*, U.S. Patent No. 5,738,846, Nieforth *et al.*, *Clin. Pharmacol. Ther.* 59:636 (1996), Monkash *et al.*, *Anal. Biochem.* 247:434 (1997).

6. *Production of Zcys8 Polypeptides in Cultured Cells*

The polypeptides of the present invention, including full-length polypeptides, functional fragments, and fusion proteins, can be produced in recombinant host cells following conventional techniques. To express a Zcys8 gene, a nucleic acid molecule encoding the polypeptide must be operably linked to regulatory sequences that control transcriptional expression in an expression vector and then, introduced into a host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors can include translational regulatory sequences and a marker gene that is suitable for selection of cells that carry the expression vector.

Expression vectors that are suitable for production of a foreign protein in eukaryotic cells typically contain (1) prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance marker to provide for the growth and selection of the expression vector in a bacterial host; (2) eukaryotic DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence. As discussed above, expression vectors can also include nucleotide sequences encoding a secretory sequence that directs the heterologous polypeptide into the secretory pathway of a host cell. For example, a Zcys8 expression vector may comprise a Zcys8 gene and a secretory sequence derived from a Zcys8 gene or another secreted gene.

Zcys8 proteins of the present invention may be expressed in mammalian cells. Examples of suitable mammalian host cells include African green monkey kidney cells (Vero; ATCC CRL 1587), human embryonic kidney cells (293-HEK; ATCC CRL 1573), baby hamster kidney cells (BHK-21, BHK-570; ATCC CRL 8544, ATCC CRL 10314), canine kidney cells (MDCK; ATCC CCL 34), Chinese hamster ovary cells (CHO-K1; ATCC CCL61; CHO DG44 [Chasin *et al.*, *Som. Cell. Molec. Genet.* 12:555 (1986)]], rat pituitary cells (GH1; ATCC CCL82), HeLa S3 cells (ATCC CCL2.2), rat hepatoma cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene which has a high level of expression. Suitable transcriptional and translational regulatory sequences also can be obtained from mammalian genes, such as *actin*,
5 *collagen*, *myosin*, and *metallothionein* genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse *metallothionein I* gene [Hamer *et al.*, *J. Molec. Appl. Genet.* 1:273 (1982)], the *TK* promoter of *Herpes* virus [McKnight, *Cell* 31:355 (1982)], the *SV40* early promoter [Benoist *et al.*, *Nature* 290:304 (1981)], the *Rous* sarcoma virus promoter (Gorman *et al.*, *Proc. Nat'l Acad. Sci. USA* 79:6777 (1982)), the cytomegalovirus promoter [Foecking *et al.*, *Gene* 45:101 (1980)], and the mouse mammary tumor virus promoter [see, generally, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163-181 (John Wiley & Sons, Inc. 1996)].

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control *Zcys8* gene expression in mammalian cells if the prokaryotic promoter is regulated by a eukaryotic promoter [Zhou *et al.*, *Mol. Cell. Biol.* 10:4529 (1990), and Kaufman *et al.*, *Nucl. Acids Res.* 19:4485 (1991)].

An expression vector can be introduced into host cells using a variety of standard techniques including calcium phosphate transfection, liposome-mediated transfection, microprojectile-mediated delivery, electroporation, and the like. Preferably, the transfected cells are selected and propagated to provide recombinant host cells that comprise the expression vector stably integrated in the host cell genome. Techniques for introducing vectors into eukaryotic cells and techniques for selecting such stable transformants using a dominant selectable marker are described, for example, by Ausubel (1995) and by Murray (ed.), *Gene Transfer and Expression Protocols* (Humana Press 30 1991).

For example, one suitable selectable marker is a gene that provides resistance to the antibiotic neomycin. In this case, selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectedants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (*e.g.*, hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternatively, markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Zcys8 polypeptides can also be produced by cultured mammalian cells using a viral delivery system. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid [for a review, see Becker *et al.*, *Meth. Cell Biol.* 43:161 (1994), and Douglas and Curiel, *Science & Medicine* 4:44 (1997)]. Advantages of the adenovirus system include the accommodation of relatively large DNA inserts, the ability to grow to high-titer, the ability to infect a broad range of mammalian cell types, and flexibility that allows use with a large number of available vectors containing different promoters.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. An option is to delete the essential *E1* gene from the viral vector, which results in the inability to replicate unless the *E1* gene is provided by the host cell. Adenovirus vector-infected human 293 cells (ATCC Nos. CRL-1573, 45504, 45505), for example, can be grown as adherent cells or in suspension culture at relatively high

cell density to produce significant amounts of protein [see Garnier *et al.*, *Cytotechnol.* 15:145 (1994)].

Zcys8 genes may also be expressed in other higher eukaryotic cells, such as avian, fungal, insect, yeast, or plant cells. The baculovirus system provides an efficient means to introduce cloned Zcys8 genes into insect cells. Suitable expression vectors are based upon the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), and contain well-known promoters such as *Drosophila heat shock protein* (*hsp*) 70 promoter, *Autographa californica nuclear polyhedrosis virus immediate-early* gene promoter (*ie-1*) and the *delayed early* 39K promoter, baculovirus *p10* promoter, and the *Drosophila metallothionein* promoter. A second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow, *et al.*, *J. Virol.* 67:4566 (1993)). This system, which utilizes transfer vectors, is sold in the BAC-to-BAC kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, PFASTBAC (Life Technologies) containing a Tn7 transposon to move the DNA encoding the Zcys8 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." See, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971 (1990), Bonning, *et al.*, *J. Gen. Virol.* 75:1551 (1994), and Chazenbalk, and Rapoport, *J. Biol. Chem.* 270:1543 (1995). In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed Zcys8 polypeptide, for example, a Glu-Glu epitope tag [Grussenmeyer *et al.*, *Proc. Nat'l Acad. Sci.* 82:7952 (1985)]. Using a technique known in the art, a transfer vector containing a Zcys8 gene is transformed into *E. coli*, and screened for bacmids that contain an interrupted *lacZ* gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is then isolated using common techniques.

The illustrative PFASTBAC vector can be modified to a considerable degree. For example, the polyhedrin promoter can be removed and substituted with the baculovirus basic protein promoter (also known as *Pcor*, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins (see, for example, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971 (1990), Bonning, *et al.*, *J. Gen. Virol.* 75:1551 (1994), and Chazenbalk

and Rapoport, *J. Biol. Chem.* 270:1543 (1995). In such transfer vector constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed that replace the native Zcys8 secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honeybee Melittin (Invitrogen Corporation; Carlsbad, CA), or baculovirus gp67 (PharMingen: San Diego, CA) can be used in constructs to replace the native Zcys8 secretory signal sequence.

The recombinant virus or bacmid is used to transfect host cells. Suitable insect host cells include cell lines derived from IPLB-Sf-21, a *Spodoptera frugiperda* pupal ovarian cell line, such as Sf9 (ATCC CRL 1711), Sf21AE, and Sf21 (Invitrogen Corporation; San Diego, CA), as well as *Drosophila* Schneider-2 cells, and the HIGH FIVEO cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent No. 5,300,435). Commercially available serum-free media can be used to grow and to maintain the cells. Suitable media are Sf900 IITM (Life Technologies) or ESF 921TM (Expression Systems) for the Sf9 cells; and Ex-cellO405TM (JRH Biosciences, Lenexa, KS) or Express FiveOTM (Life Technologies) for the *T. ni* cells. When recombinant virus is used, the cells are typically grown up from an inoculation density of approximately 2-5 x 10⁵ cells to a density of 1-2 x 10⁶ cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3.

Established techniques for producing recombinant proteins in baculovirus systems are provided by Bailey *et al.*, "Manipulation of Baculovirus Vectors," in *Methods in Molecular Biology, Volume 7: Gene Transfer and Expression Protocols*, Murray (ed.), pages 147-168 (The Humana Press, Inc. 1991), by Patel *et al.*, "The baculovirus expression system," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 205-244 (Oxford University Press 1995), by Ausubel (1995) at pages 16-37 to 16-57, by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995), and by Lucknow, "Insect Cell Expression Technology," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 183-218 (John Wiley & Sons, Inc. 1996).

Fungal cells, including yeast cells, can also be used to express the genes described herein. Yeast species of particular interest in this regard include

DRAFT 6.0
PCT/US2018/036200
100-1000

Saccharomyces cerevisiae, *Pichia pastoris*, and *Pichia methanolica*. Suitable promoters for expression in yeast include promoters from *GAL1* (galactose), *PGK* (phosphoglycerate kinase), *ADH* (alcohol dehydrogenase), *AOX1* (alcohol oxidase), *HIS4* (histidinol dehydrogenase), and the like. Many yeast cloning vectors have been 5 designed and are readily available. These vectors include YIp-based vectors, such as YIp5, YRp vectors, such as YRp17, YEp vectors such as YEp13 and YCp vectors, such as YCp19. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311, Kawasaki *et al.*, U.S. Patent No. 4,931,373, Brake, U.S. Patent No. 4,870,008, Welch *et al.*, U.S. Patent No. 5,037,743, and Murray 10 *et al.*, U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (*e.g.*, leucine). A preferred vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki *et al.* 15 (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Additional suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, *e.g.*, Kawasaki, U.S. Patent No. 4,599,311, Kingsman *et al.*, U.S. Patent No. 4,615,974, and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446, 20 5,063,154, 5,139,936, and 4,661,454.

Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson *et al.*, *J. Gen. Microbiol.* 132:3459 (1986), and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells 25 may be utilized according to the methods of McKnight *et al.*, U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino *et al.*, U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

The use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed by Raymond, U.S. Patent No. 5,716,808, Raymond, U.S. Patent 30

No. 5,736,383, Raymond *et al.*, *Yeast* 14:11-23 (1998), and in international publication Nos. WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica* *ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), and which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. *P. methanolica* cells can be transformed by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (t) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Expression vectors can also be introduced into plant protoplasts, intact plant tissues, or isolated plant cells. Methods for introducing expression vectors into plant tissue include the direct infection or co-cultivation of plant tissue with *Agrobacterium tumefaciens*, microprojectile-mediated delivery, DNA injection, electroporation, and the like. See, for example, Horsch *et al.*, *Science* 227:1229 (1985), Klein *et al.*, *Biotechnology* 10:268 (1992), and Miki *et al.*, "Procedures for Introducing Foreign DNA into Plants," in *Methods in Plant Molecular Biology and Biotechnology*, Glick *et al.* (eds.), pages 67-88 (CRC Press, 1993).

Zcys8 genes can also be expressed in prokaryotic host cells. Suitable promoters that can be used to express Zcys8 polypeptides in a prokaryotic host are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the trp, recA, heat shock, lacUV5, tac, lpp-lacSpr, phoA, and lacZ promoters of *E. coli*, 5 promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the int promoter of bacteriophage lambda, the bla promoter of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters have been reviewed by Glick, *J. Ind. Microbiol.* 1:277 (1987), Watson *et al.*, 10 *Molecular Biology of the Gene*, 4th Ed. (Benjamin Cummins 1987), and by Ausubel *et al.* (1995).

Preferred prokaryotic hosts include *E. coli* and *Bacillus subtilis*.

Suitable strains of *E. coli* include BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, DH1, DH4I, DH5, DH5I, DH5IF, DH5IMCR, DH10B, DH10B/p3, DH11S, C600, 15 HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, and ER1647 [see, for example, Brown (ed.), *Molecular Biology Labfax* (Academic Press 1991)]. Suitable strains of *Bacillus subtilis* include BR151, YB886, MI119, MI120, and B170 [see, for example, Hardy, "Bacillus Cloning Methods," in *DNA 20 Cloning: A Practical Approach*, Glover (ed.) (IRL Press 1985)].

When expressing a Zcys8 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and 25 dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space 30 and recovering the protein, thereby obviating the need for denaturation and refolding.

Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art [see, for example, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 15 (Oxford University Press 1995), Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, page 137 (Wiley-Liss, Inc. 1995), and Georgiou, "Expression of Proteins in Bacteria," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), page 101 (John Wiley & Sons, Inc. 1996)].

Standard methods for introducing expression vectors into bacterial, yeast, insect, and plant cells are provided, for example, by Ausubel (1995).

General methods for expressing and recovering foreign protein produced by a mammalian cell system are provided by, for example, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163 (Wiley-Liss, Inc. 1996). Standard techniques for recovering protein produced by a bacterial system is provided by, for example, Grisshammer *et al.*, "Purification of over-produced proteins from *E. coli* cells," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 59-92 (Oxford University Press 1995). Established methods for isolating recombinant proteins from a baculovirus system are described by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995).

7. Isolation of Zcys8 Polypeptides

It is preferred to purify the polypeptides of the present invention to at least about 80% purity, more preferably to at least about 90% purity, even more preferably to at least about 95% purity, or even greater than 95% purity with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. The polypeptides of the present invention may also be purified to a pharmaceutically pure state, which is greater than 99.9% pure.

Preferably, a purified polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin.

SEARCHED - INDEXED - COPIED - FILED

Fractionation and/or conventional purification methods can be used to obtain preparations of Zcys8 purified from natural sources (e.g., coronary artery smooth muscle tissue, placental tissue, uterine tissue, or tracheal tissue), and recombinant Zcys8 polypeptides and fusion Zcys8 polypeptides purified from recombinant host cells. In general, ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties.

Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Selection of a particular method for polypeptide isolation and purification is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, *Affinity Chromatography: Principles & Methods* (Pharmacia LKB Biotechnology 1988), and Doonan, *Protein Purification Protocols* (The Humana Press 1996).

Additional variations in Zcys8 isolation and purification can be devised by those of skill in the art. For example, anti-Zcys8 antibodies, obtained as described below, can be used to isolate large quantities of protein by immunoaffinity purification.

The use of monoclonal antibody columns to purify interferons from recombinant cells and from natural sources has been described, for example, by Staehelin *et al.*, *J. Biol. Chem.* 256:9750 (1981), and by Adolf *et al.*, *J. Biol. Chem.* 265:9290 (1990).

Moreover, methods for binding ligands, such as Zcys8, to receptor polypeptides bound to support media are well known in the art.

The polypeptides of the present invention can also be isolated by exploitation of particular properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate [Sulkowski, *Trends in Biochem.* 3:1 (1985)]. Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography [M. Deutscher, (ed.), *Meth. Enzymol.* 182:529 (1990)]. For example, the Zcys8 γ isolation method of Rinderknecht *et al.*, *J. Biol. Chem.* 259:6790 (1984), requires the binding of the interferon with concanavalin A-sepharose in one step. Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (*e.g.*, maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

Zcys8 polypeptides or fragments thereof may also be prepared through chemical synthesis, as described below. Zcys8 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; PEGylated or non-PEGylated; and may or may not include an initial methionine amino acid residue.

Peptides and polypeptides of the present invention comprise at least at least 15, preferably at least 30 or 50 contiguous amino acid residues of SEQ ID NOS: 2, 3 or 4. Within certain embodiments of the invention, the polypeptides comprise 20, 30, 40, 50, 100, or more contiguous residues of these amino acid sequences. Nucleic acid molecules encoding such peptides and polypeptides are useful as polymerase chain reaction primers and probes.

8. Assays for Zcys8, Its Analogs, and the Zcys8 Receptor

As described above, the disclosed polypeptides can be used to construct Zcys8 variants. These Zcys8 variants can be initially identified on the basis of hybridization analysis, sequence identity determination, or by the ability to specifically bind anti-Zcys8 antibody. Zcys8, its agonists and antagonists are valuable in both *in vivo* and *in vitro* uses. As an illustration, cytokines can be used as components of defined cell culture media, alone or in combination with other cytokines and hormones, to replace serum that is commonly used in cell culture. Antagonists are also useful as research reagents for characterizing sites of interaction between Zcys8 and its receptor. In a therapeutic setting, pharmaceutical compositions comprising Zcys8 antagonists can be used to inhibit Zcys8 activity.

One general class of Zcys8 analogs are agonists or antagonists having an amino acid sequence that is a mutation of the amino acid sequences disclosed herein. Another general class of Zcys8 analogs is provided by anti-idiotype antibodies, and fragments thereof, as described below. Moreover, recombinant antibodies comprising anti-idiotype variable domains can be used as analogs [see, for example, Monfardini *et al.*, *Proc. Assoc. Am. Physicians* 108:420 (1996)]. Since the variable domains of anti-idiotype Zcys8 antibodies mimic Zcys8, these domains can provide either Zcys8 agonist or antagonist activity.

20 A third approach to identifying Zcys8 analogs is provided by the use of combinatorial libraries. Methods for constructing and screening phage display and other combinatorial libraries are provided, for example, by Kay *et al.*, *Phage Display of Peptides and Proteins* (Academic Press 1996), Verdine, U.S. Patent No. 5,783,384, Kay, *et. al.*, U.S. Patent No. 5,747,334, and Kauffman *et al.*, U.S. Patent No. 5,723,323.

9 Production of Antibodies to Zcys8 Proteins

Antibodies to Zcys8 can be obtained, for example, using the product of a Zcys8 expression vector or Zcys8 isolated from a natural source as an antigen. Particularly useful anti-Zcys8 antibodies “bind specifically” with Zcys8. Antibodies are considered to be specifically binding if the antibodies exhibit at least one of the

following two properties: (1) antibodies bind to Zcys8 with a threshold level of binding activity, and (2) antibodies do not significantly cross-react with other polypeptides.

With regard to the first characteristic, antibodies specifically bind if they bind to a Zcys8 polypeptide, peptide or epitope with a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis [Scatchard, *Ann. NY Acad. Sci.* 51:660 (1949)]. With regard to the second characteristic, antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect Zcys8, but not known related polypeptides using a standard Western blot analysis. Examples of known related polypeptides are orthologs and proteins from the same species that are members of a protein family.

Anti-Zcys8 antibodies can be produced using antigenic Zcys8 epitope-bearing peptides and polypeptides. Antigenic epitope-bearing peptides and polypeptides of the present invention contain a sequence of at least nine, preferably between 15 to about 30 amino acids contained within SEQ ID NOs: 2, 3 or 4. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of the invention, containing from 30 to 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are useful for inducing antibodies that bind with Zcys8. It is desirable that the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively hydrophilic residues, while hydrophobic residues are preferably avoided). Moreover, amino acid sequences containing proline residues may be also be desirable for antibody production.

As an illustration, potential antigenic sites in human Zcys8 were identified using the Jameson-Wolf method, Jameson and Wolf, *CABIOS* 4:181, (1988), as implemented by the PROTEAN program (version 3.14) of LASERGENE (DNASTAR; Madison, WI). Default parameters were used in this analysis.

The Jameson-Wolf method predicts potential antigenic determinants by combining six major subroutines for protein structural prediction. Briefly, the Hopp-Woods method, Hopp *et al.*, *Proc. Nat'l Acad. Sci. USA* 78:3824 (1981), was first used

DRAFT - 100-1100-1100-100

to identify amino acid sequences representing areas of greatest local hydrophilicity (parameter: seven residues averaged). In the second step, Emini's method, Emini *et al.*, *J. Virology* 55:836 (1985), was used to calculate surface probabilities (parameter: surface decision threshold (0.6) = 1). Third, the Karplus-Schultz method, Karplus and Schultz, *Naturwissenschaften* 72:212 (1985), was used to predict backbone chain flexibility (parameter: flexibility threshold (0.2) = 1). In the fourth and fifth steps of the analysis, secondary structure predictions were applied to the data using the methods of Chou-Fasman, Chou, "Prediction of Protein Structural Classes from Amino Acid Composition," in *Prediction of Protein Structure and the Principles of Protein Conformation*, Fasman (ed.), pages 549-586 (Plenum Press 1990), and Garnier-Robson, Garnier *et al.*, *J. Mol. Biol.* 120:97 (1978) (Chou-Fasman parameters: conformation table = 64 proteins; α region threshold = 103; β region threshold = 105; Garnier-Robson parameters: α and β decision constants = 0). In the sixth subroutine, flexibility parameters and hydrophathy/solvent accessibility factors were combined to determine a surface contour value, designated as the "antigenic index." Finally, a peak broadening function was applied to the antigenic index, which broadens major surface peaks by adding 20, 40, 60, or 80% of the respective peak value to account for additional free energy derived from the mobility of surface regions relative to interior regions. This calculation was not applied, however, to any major peak that resides in a helical region, since helical regions tend to be less flexible.

The results of this analysis indicated that the following amino acid sequences of SEQ ID NO: 2 would provide suitable antigenic peptides: amino acids 32 - 122 (SEQ ID NO: 5), amino acids 26 to 61 (SEQ ID NO: 6), amino acids 44 to 61 (SEQ ID NO: 7), amino acids 44 to 78 (SEQ ID NO: 8), amino acids 52 to 78 (SEQ ID NO: 9), amino acids 55 to 100 (SEQ ID NO: 10), amino acids 74 to 97 (SEQ ID NO: 11), amino acids 75 to 107 (SEQ ID NO: 12), amino acids 52 to 100 (SEQ ID NO: 13), amino acids 56 to 107 (SEQ ID NO: 14), amino acids 53 to 132 (SEQ ID NO: 15), amino acids 74 to 132 (SEQ ID NO: 16) and amino acids 85 to 132 (SEQ ID NO: 17). The present invention contemplates the use of any one of antigenic peptides to generate antibodies to Zcys8. The present invention also contemplates polypeptides comprising at least one of the above-described antigenic peptides.

Polyclonal antibodies to recombinant Zcys8 protein or to Zcys8 isolated from natural sources can be prepared using methods well known to those of skill in the art. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in *Immunochemical Protocols* (Manson, ed.), pages 1-5 (Humana Press 1992), and Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 15 (Oxford University Press 1995). The immunogenicity of a Zcys8 polypeptide can be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of Zcys8 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like," such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

Although polyclonal antibodies are typically raised in animals such as horses, cows, dogs, chicken, rats, mice, rabbits, guinea pigs, goats, or sheep, an anti-Zcys8 antibody of the present invention may also be derived from a subhuman primate antibody. General techniques for raising diagnostically and therapeutically useful antibodies in baboons may be found, for example, in Goldenberg *et al.*, international patent publication No. WO 91/11465, and in Losman *et al.*, *Int. J. Cancer* 46:310 (1990).

Alternatively, monoclonal anti-Zcys8 antibodies can be generated. Rodent monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art [see, for example, Kohler *et al.*, *Nature* 256:495 (1975), Coligan *et al.* (eds.), *Current Protocols in Immunology*, Vol. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991) [“Coligan”], Picksley *et al.*, “Production of monoclonal antibodies against proteins expressed in *E. coli*,” in *DNA Cloning 2: Expression Systems*, 2nd Edition, Glover *et al.* (eds.), page 93 (Oxford University Press 1995)].

Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an *Zcys8* gene product, verifying the presence of antibody

production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

In addition, an anti-Zcys8 antibody of the present invention may be derived from a human monoclonal antibody. Human monoclonal antibodies are obtained from transgenic mice that have been engineered to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described, for example, by Green *et al.*, *Nature Genet.* 7:13 (1994), Lonberg *et al.*, *Nature* 368:856 (1994), and Taylor *et al.*, *Int. Immun.* 6:579 (1994).

Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography [see, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3; Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in *Methods in Molecular Biology*, Vol. 10, pages 79-104 (The Humana Press, Inc. 1992)].

For particular uses, it may be desirable to prepare fragments of anti-Zcys8 antibodies. Such antibody fragments can be obtained, for example, by proteolytic hydrolysis of the antibody. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As an illustration, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab' monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulphydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic

DOCUMENT NUMBER

cleavage using pepsin produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent No. 4,331,647, Nisonoff *et al.*, *Arch Biochem. Biophys.* 89:230 (1960), Porter, *Biochem. J.* 73:119 (1959), Edelman *et al.*, in *Methods in Enzymology Vol. 1*, page 422 (Academic Press 1967), and by Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of V_H and V_L chains. This association can be noncovalent, as described by Inbar *et al.*, *Proc. Nat'l Acad. Sci. USA* 69:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (see, for example, Sandhu, *Crit. Rev. Biotech.* 12:437 (1992)).

The Fv fragments may comprise V_H and V_L chains which are connected by a peptide linker. These single-chain antigen binding proteins (scFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains which are connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell, such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing scFvs are described, for example, by Whitlow *et al.*, *Methods: A Companion to Methods in Enzymology* 2:97 (1991) (also see, Bird *et al.*, *Science* 242:423 (1988), Ladner *et al.*, U.S. Patent No. 4,946,778, Pack *et al.*, *Bio/Technology* 11:1271 (1993), and Sandhu, *supra*).

As an illustration, a scFV can be obtained by exposing lymphocytes to Zcys8 polypeptide *in vitro*, and selecting antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled Zcys8 protein or peptide). Genes encoding polypeptides having potential Zcys8 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random

polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides that interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art [Ladner *et al.*, U.S. Patent No. 5,223,409, Ladner *et al.*, U.S. Patent No. 4,946,778, Ladner *et al.*, U.S. Patent No. 5,403,484, Ladner *et al.*, U.S. Patent No. 5,571,698, and Kay *et al.*, *Phage Display of Peptides and Proteins* (Academic Press, Inc. 1996)) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from CLONTECH Laboratories, Inc. (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA), and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the Zcys8 sequences disclosed herein to identify proteins that bind to Zcys8.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells [see, for example, Larrick *et al.*, *Methods: A Companion to Methods in Enzymology* 2:106 (1991), Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in *Monoclonal Antibodies: Production, Engineering and Clinical Application*, Ritter *et al.* (eds.), page 166 (Cambridge University Press 1995), and Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, Birch *et al.*, (eds.), page 137 (Wiley-Liss, Inc. 1995)].

Alternatively, an anti-Zcys8 antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementary determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain. Typical residues of human antibodies are then substituted in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity

TOP SECRET//TECHNOLOGY

of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:3833 (1989). Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* 321:522 (1986), Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:4285 (1992), Sandhu, *Crit. Rev. Biotech.* 12:437 (1992), Singer *et al.*, *J. Immun.* 150:2844 (1993), Sudhir (ed.), *Antibody Engineering Protocols* (Humana Press, Inc. 1995), Kelley, "Engineering Therapeutic Antibodies," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 399-434 (John Wiley & Sons, Inc. 1996), and by Queen *et al.*, U.S. Patent No. 5,693,762 (1997).

10 Polyclonal anti-idiotype antibodies can be prepared by immunizing animals with anti-Zcys8 antibodies or antibody fragments, using standard techniques. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in *Methods In Molecular Biology: Immunochemical Protocols*, Manson (ed.), pages 1-12 (Humana Press 1992). Also, see Coligan at pages 2.4.1-2.4.7. Alternatively, monoclonal anti-idiotype antibodies can be prepared using anti-Zcys8 antibodies or antibody fragments as immunogens with the techniques, described above. As another alternative, humanized anti-idiotype antibodies or subhuman primate anti-idiotype antibodies can be prepared using the above-described techniques. Methods for producing anti-idiotype antibodies are described, for example, by Irie, U.S. Patent No. 5,208,146, Greene, *et. al.*, U.S. Patent No. 5,637,677, and Varthakavi and Minocha, *J. Gen. Virol.* 77:1875 (1996).

10. Diagnostic Application of Zcys8 Nucleotide Sequences

25 Nucleic acid molecules can be used to detect the expression of a Zcys8 gene in a biological sample. Although such probe molecules can include murine Zcys8 encoding sequences, preferred probe molecules include double-stranded nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO: 1, or a fragment thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequence of SEQ ID NO: 1, or a fragment thereof. Probe molecules may be 30 DNA, RNA, oligonucleotides, and the like.

In a basic assay, a single-stranded probe molecule is incubated with RNA, isolated from a biological sample, under conditions of temperature and ionic strength that promote base pairing between the probe and target Zcys8 RNA species. After separating unbound probe from hybridized molecules, the amount of hybrids is detected. Illustrative biological samples include blood, urine, saliva, tissue biopsy, and autopsy material.

Well-established hybridization methods of RNA detection include northern analysis and dot/slot blot hybridization [see, for example, Ausubel (1995) at pages 4-1 to 4-27, and Wu *et al.* (eds.), "Analysis of Gene Expression at the RNA Level," in *Methods in Gene Biotechnology*, pages 225-239 (CRC Press, Inc. 1997)]. Nucleic acid probes can be detectably labeled with radioisotopes such as ^{32}P or ^{35}S . Alternatively, Zcys8 RNA can be detected with a nonradioactive hybridization method [see, for example, Isaac (ed.), *Protocols for Nucleic Acid Analysis by Nonradioactive Probes* (Humana Press, Inc. 1993)]. Typically, nonradioactive detection is achieved by enzymatic conversion of chromogenic or chemiluminescent substrates. Illustrative nonradioactive moieties include biotin, fluorescein, and digoxigenin.

Zcys8 oligonucleotide probes are also useful for *in vivo* diagnosis. As an illustration, ^{18}F -labeled oligonucleotides can be administered to a subject and visualized by positron emission tomography [Tavitian *et al.*, *Nature Medicine* 4:467 (1998)].

Numerous diagnostic procedures take advantage of the polymerase chain reaction (PCR) to increase sensitivity of detection methods. Standard techniques for performing PCR are well-known [see, generally, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), White (ed.), *PCR Protocols: Current Methods and Applications* (Humana Press, Inc. 1993), Cotter (ed.), *Molecular Diagnosis of Cancer* (Humana Press, Inc. 1996), Hanausek and Walaszek (eds.), *Tumor Marker Protocols* (Humana Press, Inc. 1998), Lo (ed.), *Clinical Applications of PCR* (Humana Press, Inc. 1998), and Meltzer (ed.), *PCR in Bioanalysis* (Humana Press, Inc. 1998)].

One variation of PCR for diagnostic assays is reverse transcriptase-PCR (RT-PCR). In the RT-PCR technique, RNA is isolated from a biological sample, reverse transcribed to cDNA, and the cDNA is incubated with Zcys8 primers [see, for

2000 RELEASE UNDER E.O. 14176

example, Wu *et al.* (eds.), "Rapid Isolation of Specific cDNAs or Genes by PCR," in *Methods in Gene Biotechnology*, pages 15-28 (CRC Press, Inc. 1997)]. PCR is then performed and the products are analyzed using standard techniques.

As an illustration, RNA is isolated from biological sample using, for example, the guanidinium-thiocyanate cell lysis procedure described above. Alternatively, a solid-phase technique can be used to isolate mRNA from a cell lysate. A reverse transcription reaction can be primed with the isolated RNA using random oligonucleotides, short homopolymers of dT, or Zcys8anti-sense oligomers. Oligo-dT primers offer the advantage that various mRNA nucleotide sequences are amplified that can provide control target sequences. Zcys8 sequences are amplified by the polymerase chain reaction using two flanking oligonucleotide primers that are typically 20 bases in length.

PCR amplification products can be detected using a variety of approaches. For example, PCR products can be fractionated by gel electrophoresis, and visualized by ethidium bromide staining. Alternatively, fractionated PCR products can be transferred to a membrane, hybridized with a detectably-labeled Zcys8 probe, and examined by autoradiography. Additional alternative approaches include the use of digoxigenin-labeled deoxyribonucleic acid triphosphates to provide chemiluminescence detection, and the C-TRAK colorimetric assay.

Another approach for detection of Zcys8 expression is cycling probe technology (CPT), in which a single-stranded DNA target binds with an excess of DNA-RNA-DNA chimeric probe to form a complex, the RNA portion is cleaved with RNAase H, and the presence of cleaved chimeric probe is detected [see, for example, Beggs *et al.*, *J. Clin. Microbiol.* 34:2985 (1996), Bekkaoui *et al.*, *Biotechniques* 20:240 (1996)]. Alternative methods for detection of Zcys8 sequences can utilize approaches such as nucleic acid sequence-based amplification (NASBA), cooperative amplification of templates by cross-hybridization (CATCH), and the ligase chain reaction (LCR) [see, for example, Marshall *et al.*, U.S. Patent No. 5,686,272 (1997), Dyer *et al.*, *J. Virol. Methods* 60:161 (1996), Ehricht *et al.*, *Eur. J. Biochem.* 243:358 (1997), and Chadwick *et al.*, *J. Virol. Methods* 70:59 (1998)]. Other standard methods are known to those of skill in the art.

Zcys8 probes and primers can also be used to detect and to localize Zcys8 gene expression in tissue samples. Methods for such *in situ* hybridization are well-known to those of skill in the art (see, for example, Choo (ed.), *In Situ Hybridization Protocols* (Humana Press, Inc. 1994), Wu *et al.* (eds.), "Analysis of 5 Cellular DNA or Abundance of mRNA by Radioactive *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 259-278 [CRC Press, Inc. 1997], and Wu *et al.* (eds.), "Localization of DNA or Abundance of mRNA by Fluorescence *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 279-289 (CRC Press, Inc. 1997)]. Various additional diagnostic approaches are well known to those of skill 10 in the art [see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Coleman and Tsongalis, *Molecular Diagnostics* (Humana Press, Inc. 1996), and Elles, *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc., 1996)].

Nucleic acid molecules comprising Zcys8 nucleotide sequences can also 15 be used to determine whether a subject's chromosomes contain a mutation in the Zcys8 gene. Detectable chromosomal aberrations at the Zcys8 gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. Of particular interest are genetic alterations that inactivate the Zcys8 gene.

Aberrations associated with the Zcys8 locus can be detected using 20 nucleic acid molecules of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, amplification-refractory mutation system analysis (ARMS), single-strand conformation polymorphism (SSCP) detection, RNase cleavage methods, denaturing gradient gel electrophoresis, fluorescence-assisted mismatch analysis (FAMA), and other genetic analysis techniques known in the art [see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Marian, *Chest* 108:255 (1995), Coleman and Tsongalis, *Molecular Diagnostics* (Human Press, Inc. 1996), Elles (ed.) *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc. 1996), Landegren (ed.), *Laboratory Protocols for Mutation Detection* (Oxford University Press 1996), Birren *et al.* (eds.), 25 30

Genome Analysis, Vol. 2: Detecting Genes (Cold Spring Harbor Laboratory Press 1998), Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics* (John Wiley & Sons 1998), and Richards and Ward, "Molecular Diagnostic Testing," in *Principles of Molecular Medicine*, pages 83-88 (Humana Press, Inc. 1998)].

5 The protein truncation test is also useful for detecting the inactivation of a gene in which translation-terminating mutations produce only portions of the encoded protein [see, for example, Stoppa-Lyonnet *et al.*, *Blood* 91:3920 (1998)]. According to this approach, RNA is isolated from a biological sample, and used to synthesize cDNA. PCR is then used to amplify the Zcys8 target sequence and to introduce an RNA
10 polymerase promoter, a translation initiation sequence, and an in-frame ATG triplet. PCR products are transcribed using an RNA polymerase, and the transcripts are translated *in vitro* with a T7-coupled reticulocyte lysate system. The translation products are then fractionated by SDS-PAGE to determine the lengths of the translation products. The protein truncation test is described, for example, by Dracopoli *et al.*
15 (eds.), *Current Protocols in Human Genetics*, pages 9.11.1 - 9.11.18 (John Wiley & Sons 1998).

20 In a related approach, Zcys8 protein is isolated from a subject, the molecular weight of the isolated Zcys8 protein is determined, and then compared with the molecular weight a normal Zcys8 protein, such as a protein having the amino acid sequence of SEQ ID NO: 2. A substantially lower molecular weight for the isolated Zcys8 protein is indicative that the protein is truncated. In this context, "substantially lower molecular weight" refers to at least about 10 percent lower, and preferably, at least about 25 percent lower. The Zcys8 protein may be isolated by various procedures known in the art including immunoprecipitation, solid phase radioimmunoassay,
25 enzyme-linked immunosorbent assay, or Western blotting. The molecular weight of the isolated Zcys8 protein can be determined using standard techniques, such as SDS-polyacrylamide gel electrophoresis.

30 The present invention also contemplates kits for performing a diagnostic assay for Zcys8 gene expression or to detect mutations in the Zcys8 gene. Such kits comprise nucleic acid probes, such as double-stranded nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO: 1, or a fragment thereof, as well as

single-stranded nucleic acid molecules having the complement of the nucleotide sequence of SEQ ID NO: 1, or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the like. Kits may comprise nucleic acid primers for performing PCR.

5 Preferably, such a kit contains all the necessary elements to perform a nucleic acid diagnostic assay described above. A kit will comprise at least one container comprising a Zcys8 probe or primer. The kit may also comprise a second container comprising one or more reagents capable of indicating the presence of Zcys8 sequences. Examples of such indicator reagents include detectable labels such as
10 radioactive labels, fluorochromes, chemiluminescent agents, and the like. A kit may also comprise a means for conveying to the user that the Zcys8 probes and primers are used to detect Zcys8 gene expression. For example, written instructions may state that the enclosed nucleic acid molecules can be used to detect either a nucleic acid molecule that encodes Zcys8, or a nucleic acid molecule having a nucleotide sequence that is
15 complementary to a Zcys8-encoding nucleotide sequence. The written material can be applied directly to a container, or the written material can be provided in the form of a packaging insert.

11. *Diagnostic Application of Anti-Zcys8 Antibodies*

The present invention contemplates the use of anti-Zcys8 antibodies to
20 screen biological samples *in vitro* for the presence of Zcys8. In one type of *in vitro* assay, anti-Zcys8 antibodies are used in liquid phase. For example, the presence of Zcys8 in a biological sample can be tested by mixing the biological sample with a trace amount of labeled Zcys8 and an anti-Zcys8 antibody under conditions that promote binding between Zcys8 and its antibody. Complexes of Zcys8 and anti-Zcys8 in the sample can be
25 separated from the reaction mixture by contacting the complex with an immobilized protein which binds with the antibody, such as an Fc antibody or *Staphylococcus* protein A. The concentration of Zcys8 in the biological sample will be inversely proportional to the amount of labeled Zcys8 bound to the antibody and directly related to the amount of free-labeled Zcys8. Illustrative biological samples include blood, urine, saliva, tissue
30 biopsy, and autopsy material.

SEARCHED
INDEXED
MAILED
FILED
RECEIVED
JULY 2003
U.S. PATENT AND TRADEMARK OFFICE

Alternatively, *in vitro* assays can be performed in which anti-Zcys8 antibody is bound to a solid-phase carrier. For example, antibody can be attached to a polymer, such as aminodextran, in order to link the antibody to an insoluble support such as a polymer-coated bead, a plate or a tube. Other suitable *in vitro* assays will be readily apparent to those of skill in the art.

In another approach, anti-Zcys8 antibodies can be used to detect Zcys8 in tissue sections prepared from a biopsy specimen. Such immunochemical detection can be used to determine the relative abundance of Zcys8 and to determine the distribution of Zcys8 in the examined tissue. General immunochemistry techniques are well established [see, for example, Ponder, "Cell Marking Techniques and Their Application," in *Mammalian Development: A Practical Approach*, Monk (ed.), pages 115-38 (IRL Press 1987), Coligan at pages 5.8.1-5.8.8, Ausubel (1995) at pages 14.6.1 to 14.6.13 (Wiley Interscience 1990), and Manson (ed.), *Methods In Molecular Biology, Vol. 10: Immunochemical Protocols* (The Humana Press, Inc. 1992)].

Immunochemical detection can be performed by contacting a biological sample with an anti-Zcys8 antibody, and then contacting the biological sample with a detectably labeled molecule that binds to the antibody. For example, the detectably labeled molecule can comprise an antibody moiety that binds to anti-Zcys8 antibody. Alternatively, the anti-Zcys8 antibody can be conjugated with avidin/streptavidin (or biotin) and the detectably labeled molecule can comprise biotin (or avidin/streptavidin). Numerous variations of this basic technique are well known to those of skill in the art.

Alternatively, an anti-Zcys8 antibody can be conjugated with a detectable label to form an anti-Zcys8 immunoconjugate. Suitable detectable labels include, for example, a radioisotope, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label or colloidal gold. Methods of making and detecting such detectably labeled immunoconjugates are well-known to those of ordinary skill in the art, and are described in more detail below. The detectable label can be a radioisotope that is detected by autoradiography. Isotopes that are particularly useful for the purpose of the present invention are ^3H , ^{125}I , ^{131}I , ^{35}S and ^{14}C .

Anti-Zcys8 immunoconjugates can also be labeled with a fluorescent compound. The presence of a fluorescently labeled antibody is determined by exposing

the immunoconjugate to light of the proper wavelength and detecting the resultant fluorescence. Fluorescent labeling compounds include fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, anti-Zcys8 immunoconjugates can be detectably labeled by coupling an antibody component to a chemiluminescent compound. The presence of the chemiluminescent-tagged immunoconjugate is determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of chemiluminescent labeling compounds include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt and an oxalate ester.

Similarly, a bioluminescent compound can be used to label anti-Zcys8 immunoconjugates of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Bioluminescent compounds that are useful for labeling include luciferin, luciferase and aequorin.

Alternatively, anti-Zcys8 immunoconjugates can be detectably labeled by linking an anti-Zcys8 antibody component to an enzyme. When the anti-Zcys8-enzyme conjugate is incubated in the presence of the appropriate substrate, the enzyme moiety reacts with the substrate to produce a chemical moiety that can be detected, for example, by spectrophotometric, fluorometric or visual means. Examples of enzymes that can be used to detectably label polyspecific immunoconjugates include β -galactosidase, glucose oxidase, peroxidase and alkaline phosphatase.

Those of skill in the art will know of other suitable labels that can be employed in accordance with the present invention. The binding of marker moieties to anti-Zcys8 antibodies can be accomplished using standard techniques known to the art. Typical methodology in this regard is described by Kennedy *et al.*, *Clin. Chim. Acta* 70:1 (1976), Schurs *et al.*, *Clin. Chim. Acta* 81:1 (1977), Shih *et al.*, *Int'l J. Cancer* 46:1101 (1990), Stein *et al.*, *Cancer Res.* 50:1330 (1990), and Coligan, *supra*.

Moreover, the convenience and versatility of immunochemical detection can be enhanced by using anti-Zcys8 antibodies that have been conjugated with avidin, streptavidin, and biotin [see, for example, Wilchek *et al.* (eds.), "Avidin-Biotin

PROOF COPY

Technology," *Methods In Enzymology*, Vol. 184 (Academic Press 1990), and Bayer *et al.*, "Immunochemical Applications of Avidin-Biotin Technology," in *Methods In Molecular Biology*, Vol. 10, Manson (ed.), pages 149-162 (The Humana Press, Inc. 1992)].

Methods for performing immunoassays are well established. See, for example, Cook and Self, "Monoclonal Antibodies in Diagnostic Immunoassays," in *Monoclonal Antibodies: Production, Engineering, and Clinical Application*, Ritter and Ladymen (eds.), pages 180-208, (Cambridge University Press, 1995), Perry, "The Role of Monoclonal Antibodies in the Advancement of Immunoassay Technology," in *Monoclonal Antibodies: Principles and Applications*, Birch and Lennox (eds.), pages 107-120 (Wiley-Liss, Inc. 1995), and Diamandis, *Immunoassay* (Academic Press, Inc. 1996).

In a related approach, biotin- or FITC-labeled Zcys8 can be used to identify cells that bind Zcys8. Such can binding can be detected, for example, using flow cytometry.

The present invention also contemplates kits for performing an immunological diagnostic assay for Zcys8 gene expression. Such kits comprise at least one container comprising an anti-Zcys8 antibody, or antibody fragment. A kit may also comprise a second container comprising one or more reagents capable of indicating the presence of Zcys8 antibody or antibody fragments. Examples of such indicator reagents include detectable labels such as a radioactive label, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label, colloidal gold, and the like. A kit may also comprise a means for conveying to the user that Zcys8 antibodies or antibody fragments are used to detect Zcys8 protein. For example, written instructions may state that the enclosed antibody or antibody fragment can be used to detect Zcys8. The written material can be applied directly to a container, or the written material can be provided in the form of a packaging insert.

12. Therapeutic Uses of Polypeptides Having Zcys8 Activity

Generally, the dosage Zcys8 administered to promote spermatogenesis (or Zcys8 analog or fusion protein) will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical

history. Typically, it is desirable to provide the recipient with a dosage of Zcys8 that is in the range of from about 1 pg/kg to 10 mg/kg (amount of agent/body weight of patient), although a lower or higher dosage also may be administered as circumstances dictate.

5 Administration of a molecule having Zcys8 activity to a subject can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection. When administering therapeutic proteins by injection, the administration may be by continuous infusion or by single or multiple boluses. Alternatively, Zcys8 can be
10 administered as a controlled release formulation. Additional routes of administration include oral, dermal, mucosal-membrane, pulmonary, and transcutaneous. Oral delivery is suitable for polyester microspheres, zein microspheres, proteinoid microspheres, polycyanoacrylate microspheres, and lipid-based systems [see, for example, DiBase and Morrel, "Oral Delivery of Microencapsulated Proteins," in
15 *Protein Delivery: Physical Systems*, Sanders and Hendren (eds.), pages 255-288 (Plenum Press 1997)]. The feasibility of an intranasal delivery is exemplified by such a mode of insulin administration [see, for example, Hinchcliffe and Illum, *Adv. Drug Deliv. Rev.* 35:199 (1999)]. Dry or liquid particles comprising Zcys8 can be prepared and inhaled with the aid of dry-powder dispersers, liquid aerosol generators, or
20 nebulizers [e.g., Pettit and Gombotz, *TIBTECH* 16:343 (1998); Patton *et al.*, *Adv. Drug Deliv. Rev.* 35:235 (1999)]. This approach is illustrated by the AERX diabetes management system, which is a hand-held electronic inhaler that delivers aerosolized insulin into the lungs. Studies have shown that proteins as large as 48,000 kDa have been delivered across skin at therapeutic concentrations with the aid of low-frequency
25 ultrasound, which illustrates the feasibility of transcutaneous administration [Mitragotri *et al.*, *Science* 269:850 (1995)]. Transdermal delivery using electroporation provides another means to administer Zcys8 [Potts *et al.*, *Pharm. Biotechnol.* 10:213 (1997)].

30 A pharmaceutical composition comprising a protein, polypeptide, or peptide having Zcys8 activity can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the therapeutic proteins are combined in a mixture with a pharmaceutically acceptable carrier. A composition is

said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers are well known to those in the art. See, for example, Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995).

For purposes of therapy, molecules having Zcys8 activity and a pharmaceutically acceptable carrier are administered to a patient in a therapeutically effective amount. A combination of a protein, polypeptide, or peptide having Zcys8 activity and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient. An inhibition of tumor growth may be indicated, for example, by a decrease in the number of tumor cells, decreased metastasis, a decrease in the size of a solid tumor, or increased necrosis of a tumor.

Indicators of viral infection inhibition include decreased viral titer, a decrease in detectable viral antigen, or an increase in anti-viral antibody titer.

A pharmaceutical composition comprising molecules having Zcys8activity can be furnished in liquid form, in an aerosol, or in solid form. Proteins having Zcys8 activity, such as human or murine Zcys8, can be administered as a conjugate with a pharmaceutically acceptable water-soluble polymer moiety, as described above. Liquid forms, including liposome-encapsulated formulations, are illustrated by injectable solutions and oral suspensions. Exemplary solid forms include capsules, tablets, and controlled-release forms, such as a miniosmotic pump or an implant. Other dosage forms can be devised by those skilled in the art, as shown, for example, by Ansel and Popovich, *Pharmaceutical Dosage Forms and Drug Delivery Systems*, 5th Edition (Lea & Febiger 1990), Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995), and by Ranade and Hollinger, *Drug Delivery Systems* (CRC Press 1996).

As an illustration, Zcys8 pharmaceutical compositions may be supplied as a kit comprising a container that comprises Zcys8, a Zcys8 agonist, or an Zcys8 antagonist (e.g., an anti-Zcys8 antibody or antibody fragment). Zcys8 can be provided

in the form of an injectable solution for single or multiple doses, or as a sterile powder that will be reconstituted before injection. Alternatively, such a kit can include a dry-powder disperser, liquid aerosol generator, or nebulizer for administration of a therapeutic polypeptide. Such a kit may further comprise written information on 5 indications and usage of the pharmaceutical composition. Moreover, such information may include a statement that the Zcys8 composition is contraindicated in patients with known hypersensitivity to Zcys8.

13. Therapeutic Uses of Zcys8 Nucleotide Sequences

10 Immunomodulator genes can be introduced into a subject to enhance immunological responses. As an illustration "immunomodulator gene therapy" has been examined in model systems using vectors that express IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-15, Zcys8 γ , tumor necrosis factor- α , or granulocyte-macrophage colony-stimulating factor (see, for example, Cao *et al.*, *J. Gastroenterol. Hepatol.* 11:1053 15 (1996), Tahara *et al.*, *Ann. N. Y. Acad. Sci.* 795:275 (1996), Rakhmiliovich *et al.*, *Hum. Gene Ther.* 8:1303 (1997), and Cao *et al.*, *Transplantation* 65:325 (1998)). In addition, a therapeutic expression vector can be provided that inhibits Zcys8 gene expression, such as an anti-sense molecule, a ribozyme, or an external guide sequence molecule. Although murine Zcys8 nucleotide sequences can be used for these methods, 20 compositions comprising human Zcys8 nucleotide sequences are preferred for treatment of human subjects.

There are numerous approaches to introduce an Zcys8 gene to a subject, including the use of recombinant host cells that express Zcys8, delivery of naked nucleic acid encoding Zcys8, use of a cationic lipid carrier with a nucleic acid molecule 25 that encodes Zcys8, and the use of viruses that express Zcys8, such as recombinant retroviruses, recombinant adeno-associated viruses, recombinant adenoviruses, and recombinant Herpes simplex viruses [HSV] [see, for example, Mulligan, *Science* 260:926 (1993), Rosenberg *et al.*, *Science* 242:1575 (1988), LaSalle *et al.*, *Science* 259:988 (1993), Wolff *et al.*, *Science* 247:1465 (1990), Breakfield and Deluca, *The New Biologist* 3:203 (1991)]. In an *ex vivo* approach, for example, cells are isolated 30

from a subject, transfected with a vector that expresses a *Zcys8* gene, and then transplanted into the subject.

To effect expression of a *Zcys8* gene, an expression vector is constructed in which a nucleotide sequence encoding a *Zcys8* gene is operably linked to a core promoter, and optionally a regulatory element, to control gene transcription. The general requirements of an expression vector are described above.

Alternatively, a *Zcys8* gene can be delivered using recombinant viral vectors, including for example, adenoviral vectors [e.g., Kass-Eisler *et al.*, *Proc. Nat'l Acad. Sci. USA* 90:11498 (1993), Kolls *et al.*, *Proc. Nat'l Acad. Sci. USA* 91:215 (1994), Li *et al.*, *Hum. Gene Ther.* 4:403 (1993), Vincent *et al.*, *Nat. Genet.* 5:130 (1993), and Zabner *et al.*, *Cell* 75:207 (1993)], adenovirus-associated viral vectors (Flotte *et al.*, *Proc. Nat'l Acad. Sci. USA* 90:10613 (1993)], alphaviruses such as Semliki Forest Virus and Sindbis Virus [Hertz and Huang, *J. Vir.* 66:857 (1992), Raju and Huang, *J. Vir.* 65:2501 (1991), and Xiong *et al.*, *Science* 243:1188 (1989)], herpes viral vectors [e.g., U.S. Patent Nos. 4,769,331, 4,859,587, 5,288,641 and 5,328,688], parvovirus vectors (Koering *et al.*, *Hum. Gene Therap.* 5:457 (1994)], pox virus vectors [Ozaki *et al.*, *Biochem. Biophys. Res. Comm.* 193:653 (1993), Panicali and Paoletti, *Proc. Nat'l Acad. Sci. USA* 79:4927 (1982)], pox viruses, such as canary pox virus or vaccinia virus [Fisher-Hoch *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:317 (1989), and Flexner *et al.*, *Ann. N.Y. Acad. Sci.* 569:86 (1989)], and retroviruses [e.g., Baba *et al.*, *J. Neurosurg* 79:729 (1993), Ram *et al.*, *Cancer Res.* 53:83 (1993), Takamiya *et al.*, *J. Neurosci. Res.* 33:493 (1992), Vile and Hart, *Cancer Res.* 53:962 (1993), Vile and Hart, *Cancer Res.* 53:3860 (1993), and Anderson *et al.*, U.S. Patent No. 5,399,346]. Within various embodiments, either the viral vector itself, or a viral particle which contains the viral vector may be utilized in the methods and compositions described below.

As an illustration of one system, adenovirus, a double-stranded DNA virus, is a well-characterized gene transfer vector for delivery of a heterologous nucleic acid molecule [for a review, see Becker *et al.*, *Meth. Cell Biol.* 43:161 (1994); Douglas and Curiel, *Science & Medicine* 4:44 (1997)]. The adenovirus system offers several advantages including: (i) the ability to accommodate relatively large DNA inserts, (ii) the ability to be grown to high-titer, (iii) the ability to infect a broad range of

mammalian cell types, and (iv) the ability to be used with many different promoters including ubiquitous, tissue specific, and regulatable promoters. In addition, adenoviruses can be administered by intravenous injection, because the viruses are stable in the bloodstream.

Using adenovirus vectors where portions of the adenovirus genome are deleted, inserts are incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene is deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell. When intravenously administered to intact animals, adenovirus primarily targets the liver. Although an adenoviral delivery system with an E1 gene deletion cannot replicate in the host cells, the host's tissue will express and process an encoded heterologous protein. Host cells will also secrete the heterologous protein if the corresponding gene includes a secretory signal sequence. Secreted proteins will enter the circulation from tissue that expresses the heterologous gene (e.g., the highly vascularized liver).

Moreover, adenoviral vectors containing various deletions of viral genes can be used to reduce or eliminate immune responses to the vector. Such adenoviruses are E1-deleted, and in addition, contain deletions of E2A or E4 [Lusky *et al.*, *J. Virol.* 72:2022 (1998); Raper *et al.*, *Human Gene Therapy* 9:671 (1998)]. The deletion of E2b has also been reported to reduce immune responses [Amalfitano *et al.*, *J. Virol.* 72:926 (1998)]. By deleting the entire adenovirus genome, very large inserts of heterologous DNA can be accommodated. Generation of so called "gutless" adenoviruses, where all viral genes are deleted, are particularly advantageous for insertion of large inserts of heterologous DNA [for a review, see Yeh. and Perricaudet, *FASEB J.* 11:615 (1997)].

High titer stocks of recombinant viruses capable of expressing a therapeutic gene can be obtained from infected mammalian cells using standard methods. For example, recombinant HSV can be prepared in Vero cells, as described by Brandt *et al.*, *J. Gen. Virol.* 72:2043 (1991), Herold *et al.*, *J. Gen. Virol.* 75:1211 (1994), Visalli and Brandt, *Virology* 185:419 (1991), Grau *et al.*, *Invest. Ophthalmol. Vis. Sci.* 30:2474 (1989), Brandt *et al.*, *J. Virol. Meth.* 36:209 (1992), and by Brown and MacLean (eds.), *HSV Virus Protocols* (Humana Press 1997).

Alternatively, an expression vector comprising a Zcys8 gene can be introduced into a subject's cells by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner *et al.*, *Proc. Nat'l Acad. Sci. USA* 84:7413 (1987); Mackey *et al.*, *Proc. Nat'l Acad. Sci. USA* 85:8027 (1988)). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Liposomes can be used to direct transfection to particular cell types, which is particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (*e.g.*, hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

Electroporation is another alternative mode of administration. For example, Aihara and Miyazaki, *Nature Biotechnology* 16:867 (1998), have demonstrated the use of *in vivo* electroporation for gene transfer into muscle.

In an alternative approach to gene therapy, a therapeutic gene may encode a Zcys8 anti-sense RNA that inhibits the expression of Zcys8. Suitable sequences for anti-sense molecules can be derived from the nucleotide sequences of Zcys8 ϵ disclosed herein.

Alternatively, an expression vector can be constructed in which a regulatory element is operably linked to a nucleotide sequence that encodes a ribozyme. Ribozymes can be designed to express endonuclease activity that is directed to a certain target sequence in a mRNA molecule (see, for example, Draper and Macejak, U.S. Patent No. 5,496,698, McSwiggen, U.S. Patent No. 5,525,468, Chowrira and McSwiggen, U.S. Patent No. 5,631,359, and Robertson and Goldberg, U.S. Patent No. 5,225,337). In the context of the present invention, ribozymes include nucleotide sequences that bind with Zcys8 mRNA.

In another approach, expression vectors can be constructed in which a regulatory element directs the production of RNA transcripts capable of promoting RNase P-mediated cleavage of mRNA molecules that encode a Zcys8 gene. According to this approach, an external guide sequence can be constructed for directing the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, which is subsequently

cleaved by the cellular ribozyme (see, for example, Altman *et al.*, U.S. Patent No. 5,168,053, Yuan *et al.*, *Science* 263:1269 (1994), Pace *et al.*, international publication No. WO 96/18733, George *et al.*, international publication No. WO 96/21731, and Werner *et al.*, international publication No. WO 97/33991). Preferably, the external
5 guide sequence comprises a ten to fifteen nucleotide sequence complementary to Zcys8 mRNA, and a 3'-NCCA nucleotide sequence, wherein N is preferably a purine. The external guide sequence transcripts bind to the targeted mRNA species by the formation of base pairs between the mRNA and the complementary external guide sequences, thus promoting cleavage of mRNA by RNase P at the nucleotide located at the 5'-side of the
10 base-paired region.

In general, the dosage of a composition comprising a therapeutic vector having a Zcys8 nucleotide acid sequence, such as a recombinant virus, will vary depending upon such factors as the subject's age, weight, height, sex, general medical condition and previous medical history. Suitable routes of administration of therapeutic vectors include intravenous injection, intraarterial injection, intraperitoneal injection, intramuscular injection, intratumoral injection, and injection into a cavity that contains a tumor.
15

A composition comprising viral vectors, non-viral vectors, or a combination of viral and non-viral vectors of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby vectors or viruses are combined in a mixture with a pharmaceutically acceptable carrier. As noted above, a composition, such as phosphate-buffered saline is said to be a
20 "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient subject. Other suitable carriers are well-known to those in the art [see, for example, *Remington's Pharmaceutical Sciences*, 19th Ed. (Mack Publishing Co. 1995), and *Gilman's the Pharmacological Basis of Therapeutics*, 7th Ed. (MacMillan Publishing Co. 1985)].
25

For purposes of therapy, a therapeutic gene expression vector, or a recombinant virus comprising such a vector, and a pharmaceutically acceptable carrier are administered to a subject in a therapeutically effective amount. A combination of
30 an expression vector (or virus) and a pharmaceutically acceptable carrier is said to be

administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient subject

When the subject treated with a therapeutic gene expression vector or a recombinant virus is a human, then the therapy is preferably somatic cell gene therapy. That is, the preferred treatment of a human with a therapeutic gene expression vector or a recombinant virus does not entail introducing into cells a nucleic acid molecule that can form part of a human germ line and be passed onto successive generations (*i.e.*, human germ line gene therapy).

DRAFT - 2000

10

14. *Production of Transgenic Mice*

Transgenic mice can be engineered to over-express the human or murine *Zcys8* gene in all tissues or under the control of a tissue-specific or tissue-preferred regulatory element. These over-producers of *Zcys8* can be used to characterize the phenotype that results from over-expression, and the transgenic animals can serve as models for human disease caused by excess *Zcys8*. Transgenic mice that over-express *Zcys8* also provide model bioreactors for production of *Zcys8* in the milk or blood of larger animals. Methods for producing transgenic mice are well-known to those of skill in the art [see, for example, Jacob, "Expression and Knockout of Interferons in Transgenic Mice," in *Overexpression and Knockout of Cytokines in Transgenic Mice*, Jacob (ed.), pages 111-124 (Academic Press, Ltd. 1994), Monastersky and Robl (eds.), *Strategies in Transgenic Animal Science* (ASM Press 1995), and Abbud and Nilson, "Recombinant Protein Expression in Transgenic Mice," in *Gene Expression Systems: Using Nature for the Art of Expression*, Fernandez and Hoeffler (eds.), pages 367-397 (Academic Press, Inc. 1999)].

15

20

25

30

For example, a method for producing a transgenic mouse that expresses a *Zcys8* gene can begin with adult, fertile males (studs) [B6C3f1, 2-8 months of age (Taconic Farms, Germantown, NY)], vasectomized males (duds) (B6D2f1, 2-8 months, (Taconic Farms)], prepubescent fertile females (donors) [B6C3f1, 4-5 weeks, (Taconic Farms)] and adult fertile females (recipients) [B6D2f1, 2-4 months, (Taconic Farms)]. The donors are acclimated for one week and then injected with approximately 8

IU/mouse of Pregnant Mare's Serum gonadotrophin (Sigma Chemical Company; St. Louis, MO) I.P., and 46-47 hours later, 8 IU/mouse of human Chorionic Gonadotropin [hCG (Sigma)] I.P. to induce superovulation. Donors are mated with studs subsequent to hormone injections. Ovulation generally occurs within 13 hours of hCG injection.

- 5 Copulation is confirmed by the presence of a vaginal plug the morning following mating.

Fertilized eggs are collected under a surgical scope. The oviducts are collected and eggs are released into urinanalysis slides containing hyaluronidase (Sigma). Eggs are washed once in hyaluronidase, and twice in Whitten's W640 medium
10 [described, for example, by Menino and O'Claray, *Biol. Reprod.* 77:159 (1986), and Dienhart and Downs, *Zygote* 4:129 (1996)] that has been incubated with 5% CO₂, 5% O₂, and 90% N₂ at 37°C. The eggs are then stored in a 37°C/5% CO₂ incubator until microinjection.

Ten to twenty micrograms of plasmid DNA containing a Zcys8 encoding sequence is linearized, gel-purified, and resuspended in 10 mM Tris-HCl (pH 7.4), 0.25 mM EDTA (pH 8.0), at a final concentration of 5-10 nanograms per microliter for microinjection
15

Plasmid DNA is microinjected into harvested eggs contained in a drop of W640 medium overlaid by warm, CO₂-equilibrated mineral oil. The DNA is drawn into an injection needle (pulled from a 0.75mm ID, 1mm OD borosilicate glass capillary), and injected into individual eggs. Each egg is penetrated with the injection needle, into one or both of the haploid pronuclei. Picoliters of DNA are injected into the pronuclei, and the injection needle withdrawn without coming into contact with the nucleoli. The procedure is repeated until all the eggs are injected. Successfully
20 microinjected eggs are transferred into an organ tissue-culture dish with pre-gassed W640 medium for storage overnight in a 37°C/5% CO₂ incubator.
25

The following day, two-cell embryos are transferred into pseudopregnant recipients. The recipients are identified by the presence of copulation plugs, after copulating with vasectomized duds. Recipients are anesthetized and shaved on the
30 dorsal left side and transferred to a surgical microscope. A small incision is made in the skin and through the muscle wall in the middle of the abdominal area outlined by

the ribcage, the saddle, and the hind leg, midway between knee and spleen. The reproductive organs are exteriorized onto a small surgical drape. The fat pad is stretched out over the surgical drape, and a baby serrefine (Roboz, Rockville, MD) is attached to the fat pad and left hanging over the back of the mouse, preventing the organs from sliding back in.

With a fine transfer pipette containing mineral oil followed by alternating W640 and air bubbles, 12-17 healthy two-cell embryos from the previous day's injection are transferred into the recipient. The swollen ampulla is located and holding the oviduct between the ampulla and the bursa, a nick in the oviduct is made with a 28 g needle close to the bursa, making sure not to tear the ampulla or the bursa.

The pipette is transferred into the nick in the oviduct, and the embryos are blown in, allowing the first air bubble to escape the pipette. The fat pad is gently pushed into the peritoneum, and the reproductive organs allowed to slide in. The peritoneal wall is closed with one suture and the skin closed with a wound clip. The mice recuperate on a 37°C slide warmer for a minimum of four hours.

The recipients are returned to cages in pairs, and allowed 19-21 days gestation. After birth, 19-21 days postpartum is allowed before weaning. The weanlings are sexed and placed into separate sex cages, and a 0.5 cm biopsy (used for genotyping) is snipped off the tail with clean scissors.

Genomic DNA is prepared from the tail snips using, for example, a QIAGEN DNEASY kit following the manufacturer's instructions. Genomic DNA is analyzed by PCR using primers designed to amplify a *Zcys8* gene or a selectable marker gene that was introduced in the same plasmid. After animals are confirmed to be transgenic, they are backcrossed into an inbred strain by placing a transgenic female with a wild-type male, or a transgenic male with one or two wild-type female(s). As pups are born and weaned, the sexes are separated, and their tails snipped for genotyping.

To check for expression of a transgene in a live animal, a partial hepatectomy is performed. A surgical prep is made of the upper abdomen directly below the zyphoid process. Using sterile technique, a small 1.5-2 cm incision is made below the sternum and the left lateral lobe of the liver exteriorized. Using 4-0 silk, a tie

is made around the lower lobe securing it outside the body cavity. Anatraumatic clamp is used to hold the tie while a second loop of absorbable Dexon (American Cyanamid; Wayne, N.J.) is placed proximal to the first tie. A distal cut is made from the Dexon tie and approximately 100 mg of the excised liver tissue is placed in a sterile petri dish.

- 5 The excised liver section is transferred to a 14 ml polypropylene round bottom tube and snap frozen in liquid nitrogen and then stored on dry ice. The surgical site is closed with suture and wound clips, and the animal's cage placed on a 37°C heating pad for 24 hours post operatively. The animal is checked daily post operatively and the wound clips removed 7-10 days after surgery. The expression level of Zcys8 mRNA is
10 examined for each transgenic mouse using an RNA solution hybridization assay or polymerase chain reaction.

In addition to producing transgenic mice that over-express Zcys8, it is useful to engineer transgenic mice with either abnormally low or no expression of the gene. Such transgenic mice provide useful models for diseases associated with a lack of Zcys8. As discussed above, Zcys8 gene expression can be inhibited using anti-sense genes, ribozyme genes, or external guide sequence genes. To produce transgenic mice that under-express the Zcys8 gene, such inhibitory sequences are targeted to murine Zcys8 mRNA. Methods for producing transgenic mice that have abnormally low expression of a particular gene are known to those in the art [see, for example, Wu *et al.*, "Gene Underexpression in Cultured Cells and Animals by Antisense DNA and RNA Strategies," in *Methods in Gene Biotechnology*, pages 205-224 (CRC Press 1997)].

An alternative approach to producing transgenic mice that have little or no Zcys8 gene expression is to generate mice having at least one normal Zcys8 allele replaced by a nonfunctional Zcys8 gene. One method of designing a nonfunctional Zcys8 gene is to insert another gene, such as a selectable marker gene, within a nucleic acid molecule that encodes murine Zcys8. Standard methods for producing these so-called "knockout mice" are known to those skilled in the art [see, for example, Jacob, "Expression and Knockout of Interferons in Transgenic Mice," in *Overexpression and Knockout of Cytokines in Transgenic Mice*, Jacob (ed.), pages 111-124 (Academic
25
30

Press, Ltd. 1994), and Wu *et al.*, "New Strategies for Gene Knockout," in *Methods in Gene Biotechnology*, pages 339-365 (CRC Press 1997)].

15. *Uses of Zcys8*

5 Zcys8 is expressed in the testis. Regulation of reproductive function in males and females is controlled in part by feedback inhibition of the hypothalamus and anterior pituitary by blood-bone hormones. Testis proteins, such as activins and inhibins, have been shown to regulate secretion of active molecules including follicle stimulating hormone (FSH) for the pituitary [Ying, *Endocr. Rev.* 9:267-93 (1988); Plant
10 *et al.*, *Hum. Reprod.* 8:41-44 (1993)]. CRES gene expression is thought to be regulated by testicular factors or hormones [Cornwall *et al.*, *Mol. Endo.* 6:1653-64 (1992)]. Thus administration of Zcy8 to a male would be useful in promoting spermatogenesis.

15 Spermatogenesis is the sequential process whereby germ cells ultimately mature into spermatozoa, herein referred to as sperm. Testis-specific factors that influence the maturation process may come directly from the Sertoli cells that are in contact with spermatogenic cells, or may be paracrine or endocrine factors. Many of the molecules produced outside the seminiferous tubules are transported into the sperm cell microenvironment by transport and binding proteins that are expressed by the Sertoli cells within the seminiferous tubules.

20 Paracrine factors that cross the cellular barrier and enter the sperm cell microenvironment include molecules secreted from cells Leydig cells. Leydig cells are located in the interstitial space found between the seminiferous tubules in the testis, and produce several factors believed to play an important role in spermatogenic cell maturation process, such as testosterone, Leydig factor, IGF-1, inhibin and activin. The
25 expression of these, and other factors, may be specific to a defined stage in the spermatogenic cycle. Zcys8 expression was not detected in epididymus or seminal vesicles suggesting that it might be expressed by interstitial cells.

30 Molecules acting in the early stages of spermatogenesis may be involved in such activities as sperm proliferation and development. Such molecules could act to enhance sperm development. CRES expression is stage-specific during spermatogenesis and is exclusively expressed by round spermatids at Stages VII-VIII

and by early elongating spermatids of Stages IX and X [Cornwall and Hann, *Mol. Reprod. Dev.* 41:37-46 (1995)]. Testatin has been suggested to play a role in tissue reorganization in early testis development and may act as a regulator of testis-specific migration of mesonephric cells (Töhönen *et al.*, *ibid.*).

Those molecules acting at a later stage in spermatogenesis may be involved in sperm motility and egg-sperm interactions. Such molecules could also act to enhance the activity of cryopreserved sperm. Assays evaluating such activities are known [Rosenberger, *J. Androl.* 11:89-96 (1990); Fuchs, *Zentralbl Gynakol* 11:117-20 (1993); Neurwinger *et al.*, *Andrologia* 22:335-9 (1990); Harris *et al.*, *Hum. Reprod.* 3:856-60 (1988); and Jockenhovel, *Andrologia* 22:171-8 (1990); Lessing *et al.*, *Fertil. Steril.* 44:406-9 (1985); Zaneveld, In Male Infertility Chapter 11, Comhaire Ed., Chapman & Hall, London (1996)].

Proteolysis-regulated testis-specific functions, including regulation of interactions between various cell types in the seminiferous tubule during spermatogenesis as well as migration of germ cells and release of spermatids [Monsees *et al.*, *Adv. Exp. Med. Biol.* 424:111-23 (1997)], suggest a role for the cystatins in the male reproduction process. Cysteine proteinases, such as cathepsins B, H, and L are expressed in fetal testis and are inhibited by family 2 cystatins (Töhönen *et al.*, *ibid.*). Cathepsin V is expressed in testis and thymus [Brömmel *et al.*, *Biochem.* 38:2377-85 (1999)]. Cathepsins are also present in latent form in the spermatozoa. Inhibitors of cysteine proteinases, such as cystatin C, which is found in abundance in testis, epididymis, prostate and seminal vesicles may play a role in controlling proteolytic activity. Cystatin-like proteins such as Zcys8 which have alterations in sequences thought to be necessary for inhibition of known proteinases may serve to inhibit known and unknown proteinases and could also serve to modulate proteinase activity in the testis. Molecules having such activities are expected to result in enhanced fertility and successful reproduction. For example, Zcys8 polypeptides may modulate seminal fluid viscosity by inhibiting enzymatic activity. Antagonists of such molecules would be useful in contraceptive applications.

Proteins of the present invention would find application in enhancing fertilization during assisted reproduction in humans and in animals. Such assisted

TOP SECRET - EYES ONLY

reproduction methods are known in the art and include artificial insemination, *in vitro* fertilization, embryo transfer and gamete intrafallopian transfer. Such methods are useful for assisting men and women who may have physiological or metabolic disorders that prevent natural conception. Such methods are also useful in animal breeding programs, such as for livestock, zoological, endangered species or racehorse breeding, and could be used within methods for the creation of transgenic animals.

Proteins of the present invention can be used to enhance sperm production, to increase the number of viable sperm in a sample, or be combined with sperm, an egg or an egg-sperm mixture prior to fertilization of the egg to enhance fertilization. The washed sperm or sperm removed from the seminal plasma used in such assisted reproduction methods has been shown to have altered reproductive functions, in particular, reduced motility and zona interaction. To enhance fertilization during assisted reproduction methods sperm is capacitated using exogenously added compounds. Suspension of the sperm in seminal plasma from normal subjects or in a "capacitation media" containing a cocktail of compounds known to activate sperm, such as caffeine, dibutyl cyclic adenosine monophosphate (dbcAMP) or theophylline, have resulted in improved reproductive function of the sperm, in particular, sperm motility and zonae penetration [Park *et al.*, *Am. J. Obstet. Gynecol.* 158:974-9 (1988); Vandervoort *et al.*, *Mol. Repro. Develop.* 37:299-304 (1993); Vandervoort and Overstreet, *J. Androl.* 16:327-33 (1995)]. Polypeptides of the present invention can be used in such methods to enhance viability of cryopreserved sperm, enhance sperm motility and enhance fertilization, particularly in association with methods of assisted reproduction.

25 15. THE USE OF ZCYS8 TO INHIBIT CANCER PROCOAGULANT PROTEIN

Zcys8 can be administered to an individual afflicted with cancer to inhibit the thrombotic events associated with cancer. Intravascular coagulation is especially an issue in acute promyelocytic leukemia. A major procoagulant protein in promyelocytic leukemia is cancer procoagulant protein (CP), a cysteine proteinase procoagulant from fetal and malignant tissues, which directly activates factor X in the absence of factor

VII. See Falanga, A. *et al*, *Blood* 92:143-151 (2001). The administration of Zcys8 to an individual can inhibit the cancer procoagulant protein and thus inhibit the intravascular coagulation associated with the malignancy.

5

16. EDUCATIONAL KIT UTILITY OF ZCYS8 POLYPEPTIDES, POLYNUCLEOTIDES AND ANTIBODIES.

Polynucleotides and polypeptides of the present invention will additionally find use as educational tools as a laboratory practicum kits for courses related to genetics and molecular biology, protein chemistry and antibody production and analysis. Due to its unique polynucleotide and polypeptide sequence molecules of Zcys8 can be used as standards or as "unknowns" for testing purposes. For example, Zcys8 polynucleotides can be used as an aid, such as, for example, to teach a student how to prepare expression constructs for bacterial, viral, and/or mammalian expression, including fusion constructs, wherein Zcys8 is the gene to be expressed; for determining the restriction endonuclease cleavage sites of the polynucleotides; determining mRNA and DNA localization of Zcys8 polynucleotides in tissues (i.e., by Northern and Southern blotting as well as polymerase chain reaction); and for identifying related polynucleotides and polypeptides by nucleic acid hybridization.

Zcys8 polypeptides can be used educationally as an aid to teach preparation of antibodies; identifying proteins by Western blotting; protein purification; determining the weight of expressed Zcys8 polypeptides as a ratio to total protein expressed; identifying peptide cleavage sites; coupling amino and carboxyl terminal tags; amino acid sequence analysis, as well as, but not limited to monitoring biological activities of both the native and tagged protein (i.e., receptor binding, signal transduction, proliferation, and differentiation) *in vitro* and *in vivo*. Zcys8 polypeptides can also be used to teach analytical skills such as mass spectrometry, circular dichroism to determine conformation, especially of the four alpha helices, x-ray crystallography to determine the three-dimensional structure in atomic detail, nuclear magnetic resonance spectroscopy to reveal the structure of proteins in solution. For example, a kit containing the Zcys8 can be given to the student to analyze. Since the amino acid sequence would be known by the professor, the protein can be given to the student as a

D
O
C
T
E
R
W
E
D
B
D
P
D

test to determine the skills or develop the skills of the student, the teacher would then know whether or not the student has correctly analyzed the polypeptide. Since every polypeptide is unique, the educational utility of Zcys8 would be unique unto itself.

The antibodies which bind specifically to Zcys8 can be used as a teaching aid to instruct students how to prepare affinity chromatography columns to purify Zcys8, cloning and sequencing the polynucleotide that encodes an antibody and thus as a practicum for teaching a student how to design humanized antibodies. The Zcys8 gene, polypeptide or antibody would then be packaged by reagent companies and sold to universities so that the students gain skill in art of molecular biology. Because each gene and protein is unique, each gene and protein creates unique challenges and learning experiences for students in a lab practicum. Such educational kits containing the Zcys8 gene, polypeptide or antibody are considered within the scope of the present invention.

15 17. Use of Antibodies to Detect colon or testis tissue

Because Zcys8 is expressed by the colon and testis, labeled antibodies to it can be used to detect and/or separate these tissues from other tissue.

20 The present invention, thus generally described, will be understood more readily by reference to the following examples, which is provided by way of illustration and is not intended to be limiting of the present invention.

Example 1

Zcys8 was discovered in a placenta cDNA library using expressed sequence tag (EST) SEQ ID NO: 18. Northern blot analysis shows that Zcys8 is expressed in colon and testis.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.